"A STUDY OF OPIATE ACTIONS ON OXYTOCIN NEURONES"

KATHRYN MARY LIDDINGTON

PH.D.

UNIVERSITY OF EDINBURGH

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STATEMENT

The studies were performed in the Department of Physiology, University Medical School, Edinburgh and at the Department of Neuroendocrinology, A.F.R.C. Institute of Animal Physiology and Genetics Research, Babraham, Cambridge. All of the work described in this Thesis was performed by the author, unless indicated otherwise and was compiled by her alone.

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K.M. Liddington

(Nee Pumford)

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The Thesis is dedicated to my parents who have long encouraged me not only academically but in many other ways and always by their loving example.

"...I have learnt that we are all 'addicts', the only difference

is that whilst they may be indulging in hard drugs,

some of us are addicted to work, wrong relationships,

alcohol, material possessions and so on.

But we all need the grace of God in our lives."

Ah Li

(Taken from: Jackie Pullinger, 1989 "Crack in the Wall:
the life and death of Kowloon walled city.")

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ABSTRACT

The Thesis describes investigations of the interactions between opiate drugs and oxytocin neurones in the hypothalamic supraoptic nucleus (SON) of the female albino rat. Both acute and chronic opiate effects were examined, acting at the μ- and κ-subtypes of opiate receptor using whole animal and in vitro techniques.

The tolerance which develops to the chronic intracerebroventricular (i.c.v.) infusion of morphine was quantified in terms of firing rate of identified oxytocin neurones in vivo. We contrasted the attenuation of acute morphine-induced inhibition of firing rate, presumably through the μ-type opiate receptor, with the acute effects of the selective κ-type receptor agonist, U50,488H. There was no cross-tolerance between morphine and U50,488H. In both cases naloxone, the broad spectrum opiate antagonist evoked a reversal of opiate inhibition of activity with overshoot, indicating withdrawal excitation due to dependence.

The density of κ-receptors in the SON was assessed by autoradiography after chronic i.c.v. infusion of morphine, to see if the manifestations of tolerance in terms of neuronal activity might be reflected in a change in receptor density in the SON. Highly selective ligands for the μ- and δ-subtypes of opiate receptor were used to saturate binding to these two receptor subtypes, thus allowing measurement of κ-receptor density by displacement of a high affinity radio-labelled ligand with the equivalent unlabelled ligand. Tolerance to morphine did not produce a measurable change in the density of κ-opiate receptors in the SON.

The roles of two post-receptor systems in the cell response to opiates were investigated. Using i.c.v. pertussis toxin (PT) to inactivate the regulatory GTP-binding proteins (G-proteins) susceptible to it, Gi and Go, we tested the potency of morphine to inhibit the electrical activity of identified OT neurones of the SON, in vivo. Morphine inhibited firing rate less potently after PT but U50,488H remained as potent as in the i.c.v. vehicle-injected group. The G-proteins Gi or Go are involved therefore in post-receptor transduction in these cells or in cells projecting to the SON

which might mediate the acute effects of morphine.

Adenylate cyclase (AC) activity measured in vitro as cAMP content of isolated SON was assessed in SON taken from morphine-naive and chronic morphine-infused rats. Whereas vasoactive intestinal peptide (VIP) was able to powerfully stimulate AC, incubation in vitro with morphine did not affect tissue content of cAMP and treatment of SON derived from chronic morphine-infused rats with naloxone did not result in any change in tissue cAMP. cAMP appears to play no part in post-receptor events following the activation of the opiate receptor in OT neurones of the SON.

Finally we investigated the contribution of the region anterior and ventral to the third ventricle (the AV3V region) to the full expression of the withdrawal hyperexcitation of OT neurones and resultant massive and sustained release of OT into the systemic circulation. In chronic morphine infused rats, we studied plasma OT concentration, assayed before and at intervals after an electrolytic lesion of the AV3V region which left the SON and PVN intact. The withdrawal response was diminished but was still evident in AV3V-lesioned rats. So only part of the withdrawal response to naloxone is mediated outside the SON itself in the AV3V region. This suggests that SON oxytocin neurones themselves may develop morphine dependence.

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INTRODUCTION

I.1. OPIOIDS: AN HISTORICAL PERSPECTIVE

Opium occurs naturally as the exudate of the unripe seed capsules of the opium poppy, *Papaver somniferum*, indigenous to Asia Minor. Opium contains over 20 alkaloids, 4 of which have been exploited medicinally - the phenanthrene derivatives morphine and codeine and the benzylisoquinoline derivatives, papaverine and noscapine.

The psychological and medicinal properties of opium were known in the Ancient world. Sumerian ideograms in 4000B.C. describe the poppy as the 'plant of joy' with narcotic* power (*derived from the Greek word meaning drowsiness or stupor). Ancient Egyptian children were passified with an opium-containing draught. The use of opium is detailed in the medicinal records of the Babylonian king Asurbanipal, c. 700 B.C.. Greek classical literature too makes frequent reference to the ingestion of opium - Homer told of the 'cup of Helen' taken to induce forgetfulness, which was laced with the drug; Hippocrates, Virgil and Plutarch wrote of its hypnotic powers. Theophrastus recorded in his extensive botanical writings details of growing and harvesting the opium poppy.

In the 1st century A.D. Dioscorides who wrote *De Materia Medica*, (the leading work on pharmacology for some centuries), described opium. During the 5th to 10th centuries A.D. Arabic medical wisdom percolated through Europe as a result of the closer contact between Moslems and Christians with the advent of the Crusades. Along with this came the promotion of opium as a panacea, not only for ailing men but also for animals suffering the rigors of long treks. Opium was originally taken by mouth either with food or in drink but was not smoked in this region until the practice spread from China in the 19th century where it became popular after the discovery of America and the advent of pipe tobacco smoking. The use of opium became even more widespread it is thought with the prohibition of alcohol by the Prophet

Mohammed and opium addiction accompanied subsequent Moslem migrations and trade routes. Arabian traders introduced opium to India and China. Chinese medical texts as early as 1000A.D. describe it as a cure for diarrhoea. In the East, opium took on, in addition to its role a universal medicine, an important social use, akin to that of alcohol in the West, because of the powerful feeling of well-being induced in its users.

The Swiss physician, Paracelsus, in the early 16th century extolled the properties of opium so wholeheartedly that any preparation containing it was called *laudanum* (derived from the Latin *laudare*, to praise). Thomas Sydenham, a great English physician introduced opium to Britain (about 1680) and said of it, "...among the remedies which it has pleased Almighty God to give to man to relieve his sufferings, none is so universal and so efficacious as opium". As the causes of so many illnesses were a mystery to the physicians of the day, the thrust of medicine was to alleviate symptoms of disease and in this respect opium was unmatched by other available treatments. Most doctors approved enthusiastically of opium to the extent that a high proportion became addicts as did their wives.

Towards the end of the 17th century, opium smoking became popular in China as tobacco smoking was introduced. A mixture of tobacco and opium might be smoked as a treatment for malaria, but this led in time to the smoking of opium alone, largely for non-medical reasons. The last Emporer of the Ming Dynasty, Ch'ung Ch'en prohibited the smoking of tobacco and opium but both practices were continued, especially in the southern provinces where they were smuggled in by British and Portugese merchants. The East India Company monopolised the trade in opium through the port of Canton and despite a reinforcement of the Chinese ban on importation of opium, trade continued to grow. The seizure and destruction of shipments of opium in 1839 by the Chinese in an attempt to enforce their prohibition led to British intervention and the First Opium War. The defeated Chinese lost sovereignty of five Chinese ports and of Hong Kong, which were opened for trade and settlement by foreigners. A second engagement culminated in the Treaty of Tientsin

and the legalisation allowing importation of opium into China. As imports continued to grow, the Chinese government eventually decided to cultivate the poppy themselves and it began to replace cereal crops on a large scale.

In 1906 a campaign to abolish opium use was begun, involving limiting import, with the help of the British, and reclaiming land for cereal production. With the establishment of the First Republic of China in 1912, the importation, production and use of opium became illegal and by 1917 the illegal consumption of opium was almost eradicated. With the next thirty years of political upheaval in China came the re-establishment of the opium problem, accompanied by a new interest in morphine and diamorphine. The turning point for the opiate problem in China came with the creation of the Peoples's Republic of China in 1949. Dealers in opiates were persecuted and addicts rehabilitated whilst farmers were encouraged with financial incentives to switch to the cultivation of alternative crops. (The sources of much of this background information were, Bowman and Rand, 1980 and Latimer and Goldberg, 1981).

Thus far, opium had been ingested as the crude form of the drug itself, or as a laudanum. In 1803, Durosne and in 1805, a German pharmacist, Serturner isolated the main alkaloid in opium and named it *morphia* after morpheus, the Greek God of dreams. Robiguet extracted noscapine in 1817 and codeine in 1832; thebaine was isolated in 1835 by Pelletier and papaverine by Merck in 1848. Soon pure alkaloids would replace the preparations of opium in medicinal use.

Until the 20th century, opium and laudanum were widely available, not only from apothecaries but from general stores and opium in some form was an essential component of the well-stocked medicine chest. Although the opium poppy was grown in Britain briefly, the climate did not support its successful cultivation and British laudanum was not generally used (Berridge, 1977). Opium was available mostly as the laudanum but also as the camphorated tincture (paregoric elixir) and raw opium as pills or penny sticks. Many remedies contained opium and even so called 'cures' for

addicts to opium almost always contained the drug of addiction. The growing problem of misuse of opium and of morphine, the most powerful derivative of opium, was aggravated by the development of the hypodermic syringe by Dr Alexander Wood of Edinburgh. The subcutaneous administration of morphine was far more controlled and immediate than the enteral route, giving maximum intensity of effect rapidly. The parenteral route of administration was not thought to give rise to addiction as morphine does not enter the stomach where the 'appetite' for the drug was thought to arise. In fact, this method of morphine administration is especially liable to lead to addiction. The wife of Dr Wood was the first individual recorded to have died from the effects of opium addiction due to the hypodermic administration of morphine.

The misguided confidence of physicians in the safety of this mode of administration saw the distribution of hypodermic syringes to patients, with instructions and the freedom to administer morphine when required. The rapidity of effect and the much greater euphoria experienced using his method led to addicts converting to injection of opiates. In Europe such demand was created for injection apparatus that kits were manufactured in gold and silver, often richly decorated with gemstones.

In 1880, H.H. Kane in, "The Hypodermic Injection of Morphia" said, "There is no proceeding in medicine that has become so rapidly popular; no plan of medication that has been so carelessly used and thoroughly abused; and no therapeutic discovery that has been so great a blessing and so great a curse to mankind as the hypodermic injection of morphia".

This method of administration of morphine was practiced for a further 10 or 15 years before the danger became apparent. Just as the early Arabian armies were plagued by the use of opium, so many years later were the Eurpoean and American armies vulnerable, but much more so, during the Franco-Prussian War and the American Civil War. For now the much more potent morphine could be easily injected to combat disease and to treat injury, giving rise to the "army disease" -

widespread opiate addiction. In the USA the pharmaceutical industry produced morphine for parenteral administration in enormous quantities and soldiers were given syringes and a supply of morphine to administer *ad libitum* leading to a significant dependence problem. A further stimulus to the emergent opiate problem in the USA was that of a rapidly expanding population whose growth and dispersion was not matched by a supply of qualified medical practitioners and pharmacists resulting in the widespread use of proprietary medicines, many of which contained opiates. The incidence of opiate dependence grew dramatically but it was not until 1890 that an attempt at control was made by imposition of a tax and in 1909, a prohibition of opium smoking was introduced. Legislation in the form of the Narcotic Act of 1914 imposed tight controls on drugs deemed to be addictive but the drug problem was not conquered and illegal trade in opiates continued to flourish. In the late 1960s habitual users of diamorphine (heroin) were estimated at 0.1% of the population.

In the United Kingdom, opium was widely available from apothecaries, pharmacists and physicians but the spread of opium use through patent medicines was not as prevalent as in the USA, so dependence was not a problem of the same magnitude. The Dangerous Drugs Act of 1920 aimed to control the importation of opium and the manufacture and distribution of opium products but the continued dispensing of opiates to addicts by the medical profession was not interfered with, unlike the situation in the USA where physicians supplying opiates to addicts to prevent the onset of withdrawal were imprisoned. Opiate dependence was not a serious problem in Britain until the mid 1960s when the parenteral use of diamorphine became the most prevalent form of opiate drug abuse. Since then there has been a decline in the number of opiate addicts.

Chemical modification of the opium alkaloids yielded synthetic congeners often heralded as morphine substitutes, free of serious side effects, most importantly, the potential for addiction. In 1898, diamorphine (heroin), acetylated at both -OH groups of morphine was synthesised by Dreser. It shares the euphoric, analgesic, sedative and

resiratory depressant actions of morphine and in the body is converted to morphine. It is thought chiefly to act through the morphine produced upon its catabolism. It was introduced as a cough remedy and as a cure for morphine addiction. However, it turned out to be more addictive than morphine and became the drug of choice for many addicts. The allure of heroin lies partly in its greater lipid solubility which enables the user to absorb the drug simply by sniffing the powder through the nostrils. This has attracted many for whom the use of a hypodermic syringe was a disincentive. As well as reputedly producing a more intense eurphoria than morphine, the accompanying vomiting and constipation are less serious than experienced with morphine.

Pethidine, synthesised in the late 1930s was hoped to be of use as a spasmolytic, but pharmacological screening uncovered analgesic and other morphine-like properties. It was the first wholly synthetic analgesic which could replace morphine clinically. It is less potent than morphine with a shorter duration of action and is still used widely in obstetrics because of its short half-life. Like morphine it gives rise to tolerance and dependence and rapid withdrawal leads to anxiety, depression and cramps and at higher initial doses, confusion, hallucination and convulsion. The depression is an effect of pethidine whilst the central nervous system stimulation occurring at higher doses is the result of norpethidine, the demethylated metabolite of pethidine. However, pethidine has never been significantly abused.

The synthesis of methadone grew out of examination of the structural similarities between morphine and pethidine. Although a more powerful analgesic than morphine, methadone also produces euphoria, tolerance and psychological and physical dependence. The withdrawal symptoms are less severe than with morphine but are more prolonged. It is as effective orally as parenterally and morphine and methadone show cross-tolerance. For these reasons methadone was chosen as a morphine substitute in the treatment of morphine and heroin addicts. However, methadone itself has become a popularly abused drug.

(Much of this information was drawn from Latimer and Goldberg, 1981).

I.2. The chemistry and structure of opiates/opioids

Although originally the term 'opiates' was applied to drugs with a similar action to morphine that are derived from the opium poppy, or their derivatives, and 'opioids' was used to describe those morphine-like drugs produced completely synthetically, the two terms are now used interchangeably.

The structure of morphine as given below was proposed by Gulland and Robinson in 1925. The alkaloid morphine is a substituted phenanthrene molecule containing importantly, a piperidine ring which is common to morphine-like molecules. Morphine is biologically active only as the naturally occurring *laevo*-isomer. The morphine molecule has been modified to produce other opiates, as listed in Table I.2.2.. Some deserve to be highlighted. Most significant perhaps is diacetylmorphine, or heroin which is formed by the acetylation of the two -OH groups of morphine (see Figure I.2.1.). Codeine although naturally occurring is not found in sufficient quantities to meet its wide demand and is therefore prepared by the methylation of morphine (see Figure I.2.1.). Etorphine (M99; not shown in Table I.2.2.) is a derivative of morphine which is prepared from the naturally occurring thebaine and is 1,000-10,000 x more potent than morphine. The opiate antagonist (see later for background) naloxone is also derived from the modified morphine molecule.

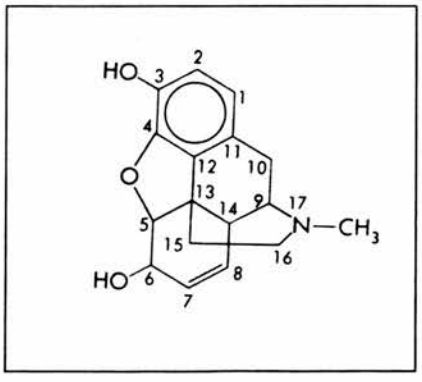


Figure I.2.1. The chemical structure of morphine

Table I.2.2. Chemical structures of some congeners of morphine

Generic name	Substitution at carbon atom number:		
	3	6	17
Morphine	-OH	-OH	$-CH_3$
Diacetylmorphine	$-OCOCH_3$	$-OCOCH_3$	$-CH_3$
Codeine	$-OCH_3$	-OH	$-CH_3$
Naloxone	-OH	=O	$-CH_2CH=CH_2$

Table I.2.2. The carbon atom number for substitutions refers to Figure I.2.1. depicting the morphine molecule with all carbon atoms numbered. The opiate antagonist naloxone has a single rather than a double bond between C7 and C8 of Figure I.2.1. (Adapted from Jaffe and Martin, 1975).

I.3. The pharmacological profile of acute opiates

Opioids share a similar pharmacological profile which I will illustrate with that of morphine, the principle opiate used in this study. Primarily, morphine affects the central nervous system (CNS) and the gastrointestinal system (GI system).

I.3.1. The effects of morphine on the CNS

Morphine is a major analgesic with its site of action in the CNS. This analgesia occurs without loss of consciousness and may be very profound or might be incomplete but the accompanying perception of pain is often lost. Other effects include drowsiness, changes in mood and mental clouding. Euphoria can occur but so can dysphoria in normal pain-free subjects with nausea and occasional vomiting. It is this euphoria which has led to the use of opiates for pleasure. After intravenous injection of morphine, the habitual user experiences an intense physical pleasure followed by a feeling of relaxation and social ease. At higher doses sedation predominates with daydreaming and fantasising. Libido and aggression are diminished and there is disinterest in the environment and sleep.

I.3.2. The effects of morphine on the GI system

Opium was used for relief of gastrointestinal complaints such as diarrhoea and dysentry, centuries before its analgesic potency was recognised. Propulsive contractions of the small intestine are markedly reduced by morphine. In the colon, peristalsis is abolished and tone increased and finally the tone of the anal sphincter is increased. These factors result in constipation being a common feature of morphine administration. These effects are not subject to tolerance (see below).

I.3.3. Other effects of morphine

Morphine is a powerful depressant of respiration by its effect on the respiratory centres in the brain stem. Death after morphine is almost always the result of this effect.

I.4. Tolerance to and dependence upon morphine

In the preceding Introduction I outlined the very gradual growth of awareness of the effects of exposure to chronic opiates and the attempts then made to legislate against opiate abuse and to develop non-addictive alternatives for medicinal use. In the next section I will provide definitions for some terms commonly used in this Thesis.

Tolerance: a state which can be both physiological and psychological, in which, "...an organism is less susceptible to the effect of drug as a consequence of its prior administration. ...Tolerance to narcotic analgesics is the alteration in response (change in character or intensity) induced by prior administration of the drug which either directly or indirectly contributes to or sustains drug-seeking behaviour." (Martin and Sloan, 1977). This can be broadened to encompass tolerance exhibited to opiates at the cellular level (see later Introduction) as follows: the state produced by prior exposure (usually repeated) to an opiate which involves adaptation of cellular processes initially affected (perhaps inhibited) by exposure to the opiate such that cell function appears as normal. The responsiveness of the cell to subsequent exposure to the same* drug however is attenuated and a higher dose of opiate is required to achieve the same magnitude of response as was achieved with the initial dose (*see the

definition of *cross-tolerance*).

Tolerance differs from *tachyphylaxis* only in the time scale over which it occurs and there is no sharp separation of the two phenomena. Tolerance which is termed tachyphylaxis is that which occurs over a relatively short time span and after less frequent exposure to the drug. The loss of effect of opiates both in vivo and in vitro is usually thought of as tolerance and not tachyphylaxis.

Cross-tolerance describes the state in which tolerance induced by one drug has conferred tolerance to another drug to which the organism (or cell) has not previously been exposed. The second drug does not necessarily possess a similar molecular structure to the first but usually has an affinity for the same type (or sub-type) of receptor* and shares a similar pharmacology (*see later Introduction). Cross-tolerance is often thought to indicate that a common receptor binding site exists for the two drugs. Because morphine or diacetylmorphine (heroin) addicts are cross-tolerant to methadone, another opiate, methadone can substitute for morphine during morphine withdrawal (see below), alleviating the withdrawal syndrome; methadone has a much less intense withdrawal syndrome than that of morphine.

Physical dependence upon a drug is not apparent until the drug is withdrawn when symptoms of a *withdrawal syndrome* can be observed. If the drug is withdrawn by administration of a competitive receptor antagonist, this is referred to as *precipitated withdrawal.*

Tolerance to opiates in man leads to the use of increasing doses to achieve the same analgesic or euphoric effect as initial doses produced. Systematic study of the phenomenon of tolerance to opiates in man was made only in 1929-1930 (Light et al, 1929-1930) even though it was clinically recognised during the 19th century. For the morphine addict heart rate, blood pressure and respiration fell within normal bounds. During withdrawal, a variety of symptoms were commonly observed. These included yawning, lacrimation, mydriasis (dilatation of the pupil), chills, piloerection, perspiration, muscle twitching, weakness, hyperthermia and an increase in metabolic

rate (Light and Torrance, 1929). The withdrawal syndrome is complex and varies subtly between opiates. Himmelsbach studied the withdrawal syndromes associated with a variety of opiates as well as morphine and found that the manifestations of withdrawal returned to baseline levels at different rates according to the opiate used and that full recovery might take 6 months (Himmelsbach, 1942).

Tolerance to and dependence upon opiates have been demonstrated in laboratory animals and it is in the animal model that headway has been made in attempting to define the mechanisms underlying the development of opiate tolerance and dependence. Tolerance has been demonstrated in terms of neuronal function at several different levels of the nervous system, both peripheral and central (Martin and Sloan, 1977). The diencephalon appears to be pivotal in expression of the behavioural signs of withdrawal. The limbic system may mediate some acute psychological features of withdrawal (Martin and Sloan, 1977). Until experiments were able to focus on in vitro preparations including individual neurones, it proved difficult to assess the contribution of putative cellular mechanisms underlying tolerance and dependence.

The fact that morphine is only active as the naturally occurring *laevo*-isomer suggested that it acts at stereospecific receptor sites to have its biological effect. Indeed an opiate binding site was discovered which was saturable and for which opiates bound competitively according to their potency (Goldstein et al, 1971; Pert and Snyder, 1973; Simon et al, 1973; Terenius, 1973) in nervous tissue. The discovery of opiate receptors provided a new starting point for future studies of mechanisms of tolerance and dependence. Subtypes of opiate receptor were first postulated by Martin in 1967 to account for the action of nalorphine in man. It antagonised morphine whilst being analgesic itself (Martin 1967). Also, some opiates fail to show full cross-tolerance and have different binding distributions in the same tissue (Chang et al, 1979; Robson and Kosterlitz, 1979; Wuster et al, 1980; Zukin and Zukin, 1981; Goodman and Snyder, 1982). Multiple opiate receptors described as mu, delta, kappa, sigma and epsilon were proposed as a result of these findings

(Martin et al, 1976; Lord et al, 1977; Schulz et al, 1979; Chang and Cuatrecasas, 1981; Pfeiffer and Herz, 1981; Zukin and Zukin, 1981). The race to understand the mechanisms of opiate tolerance and dependence was now accompanied by the hope of finding a subtype of opiate receptor which would mediate the analgesic effects of morphine but without the potential for dependence. Agonists at the mu- (μ), kappa- (κ) and delta-(δ) subtypes of opiate receptor however all develop tolerance and dependence and each have their distinct withdrawal syndromes (Redmond and Krystal, 1984). In this study it was the intention to look only at the μ- and κ-subtypes of opiate receptor in oxytocin neurones of the supraoptic and paraventricular nuclei: morphine was used to activate the μ-receptor and U50,488H (*trans*-($\pm$)-3,4-dichloro-*N*-methyl-*N*-(2-[1-pyrrolidinyl]cyclohexyl)benzeneacetamide, methane sulphonate salt) to activate the κ-receptor. Morphine is an avid ligand for the m-receptor with little relative affinity for the κ- or δ-subtype of opiate receptor (Kosterlitz, 1985). U50,488H is a high affinity ligand for the κ-receptor with a low relative affinity for the μ- or δ-subtype of opiate receptor (Kosterlitz, 1985; Lahti et al, 1985; Clarke and Pasternak, 1988).

Suggested mechanisms of tolerance and dependence have included changes in opiate receptor number, conformation or function (Collier, 1965; see Chapter 5), modulation of intracellular second messenger systems (West and Miller, 1983; Redmond and Krystal, 1984; see Chapter 4), regulation of the availability or the state of regulatory GTP-binding proteins (G-proteins; Crain et al, 1986; Nestler et al, 1989; see Chapter 3) or changes in neuronal intracellular calcium (West and Miller, 1983; Redmond and Krystal, 1984). Some of these putative mechanisms have been investigated in this Thesis in terms of both acute and chronic morphine action upon supraoptic oxytocin neurones in the rat and full experimental details are contained herein.

After the discovery of the opiate receptor, endogenous opioids were sought after with the expectancy that where there are receptors, with a distribution in the CNS

which is far from random, there must be endogenous ligands to activate them. In 1975, John Hughes, Hans Kosterlitz and colleagues reported the existence of two pentapeptides isolated from pig brain which possessed naloxone-reversible, morphine-like qualities in guinea-pig isolated ileum in vitro (Hughes et al, 1975). The two opioid peptides were named [Leu]enkephalin (Tyr-Gly-Gly-Phe-Leu) and [Met]enkephalin (Tyr-Gly-Gly-Phe-Met), distinguished only by the presence of a terminal leucine or a methionine amino acid residue. Also in 1975, Goldstein and coworkers discovered a peptide in bovine pituitary gland with opioid qualities (Cox et al, 1975; Teschemacher et al, 1975). This was in fact a 31 amino acid polypeptide and its actions were antagonised competitively by naloxone. It was designated β-endorphin. β-endorphin is derived from the carboxy terminus of another pituitary hormone, β-lipotropin (β-LPH) and [met]enkephalin corresponds to the first 5 amino acids of β-endorphin. [met]enkephalin is a product of a longer peptide, proenkephalin A. [leu]enkephalin may be derived from β-LPH or form proenkephalin B. Another peptide derived from proenkepahlin B is dynorphin which exists as dynorphin A (1-17), dynorphin A (1-8) and dynorphin B. Truncated versions of dynorphin include α-neo endorphin and β-neo endorphin. These peptides bind with differing affinities to the three principle opiate receptors (Kosterlitz, 1985). None of the endogenous opioid peptides binds with such high relative affinity to any one opiate receptor as some of the synthetic opiates are known to. Perhaps their selectivity in vivo is determined as much by the presence and distribution of receptor subtypes in a tissue as by relative affinity of the endogenous ligand. Endogenous opioid peptides are found in the oxytocin neurosecretory system, both in the magnocellular nuclei and in the posterior pituitary gland (see Introduction below). Endogenous opioid peptides have even been colocalised within the same neurone with oxytocin or vasopressin and have been implicated in the control of posterior pituitary hormone secretion, both centrally and peripherally (Lincoln and Russell, 1986). Here are circumstances then which have created immense interest in terms of the potential interaction between endogenous

opioid peptides and the mechanisms which regulate posterior pituitary hormone secretion. This hypothalamo-neurohypophyseal neurosecretory system offers a window to investigate how opiates induce tolerance and dependence in a well defined system.

I.1. OXYTOCIN: AN HISTORICAL PERSPECTIVE

Whilst the discovery of the vasopressor activity contained in the neurohypophysis had already been made before the end of the 19th century (Oliver and Schafer, 1895) the oxytocic (*οξυς*, quick, *τοκυς*, birth) potency of pituitary extract was not noted until 1906 by Sir Henry Dale. He was working at the Wellcome Physiological Research Laboratories on the pharmacology of ergot, a drug widely used at the time in obstetrics. During an experiment investigating the reversal by ergot of the effects of adrenaline and of sympathetic stimulation in the spinal cat, he controlled these experiments by testing the potency of ergot to reverse the known pressor effect of pituitary extract. As well as blood pressure, he was also monitoring the tone and rhythm of the uterus and so not only did he detect the usual pressor action of pituitary extract, unaffected by pretreatment with ergot, but he also observed powerful stimulation of the uterine muscle (Dale, 1906) thanks to chance rather than design as he later recalled (Dale, 1953). He went on however to systematically investigate the oxytocic activity in pituitary extract (Dale, 1909) and it became clear that oxytocic activity was present in the posterior lobe of the pituitary together with the pressor activity. The promotion of contraction of the alveoli of the lactating mammary gland (milk-ejection) by extracts of posterior pituitary further stimulated interest in the active principle(s) contained in the neural lobe (Ott and Scott, 1910). Whether a single agent accounted for both the pressor and the oxytocic activity of pituitary extract became a controversial question after the publication of Dale's observations and remained so even after the separation of two factors from posterior pituitary

extract with distinct pharmacologies (Dudley, 1919) which were later shown to be susceptible to proteolytic enzyme degradation and therefore probably to be peptidergic in nature (Dale and Dudley, 1921). Sharpey-Schafer addressed this point of disagreement, still current some years after the publication of the above paper (Sharpey-Schafer, 1926): comparative studies in birds, fish, amphibians and mammals revealed that pressor and oxytocic activities did not always coexist in extracts of the posterior pituitary. Herring suggested from observations in bovine posterior pituitary that the two autacoids were largely separated into the pars intermedia (containing oxytocic activity) and the pars nervosa (containing pressor activity). Herring concluded that there were two active components of posterior pituitary extract, one acting on the uterus and mammary gland and the other on blood pressure and the kidney. He thought that both were produced by the epithelium of the pars intermedia and that the pressor factor was transformed as it passed to the pars nervosa. Sharpey-Schafer upon reviewing this and other evidence concluded, '...it seems probable that the various physiological effects of extracts of posterior lobe must be caused by more than one autacoid, possibly several.' and with informed judgement later to be borne out, 'That it has not been possible to effect a chemical isolation is hardly surprising seeing that such bodies would be separated out together by most precipitation agents, for it is probable there is not much dissimilarity in their chemical constitution.'.

Upon publication of Dale's observations the potential usefulness of pituitary extract in various branches of medicine, but particularly obstetrics was recognised, and posterior pituitary extract was quickly adopted as a tool in controlling post-partum haemorrhage and later in smaller doses in cases of delayed labour (see Robinson and Amico, 1985) . Pituitary extract was recommended for intestinal stasis, particularly that experienced after surgery and whole pituitary gland given by mouth was used for treating habitual constipation (Sharpey-Schafer, 1926). These early commercial extracts of pituitary suffered contamination with histamine, which in fact appears to

have been the effective agent for intestinal complaints.

Work continued to isolate the active principles in posterior pituitary extract and in 1928 Kamm and coworkers succeeded in isolating oxytocic and pressor agents with high activity (Kamm et al, 1928) which were later purified to yield analytically-pure oxytocin by chromatography (Livermore and Du Vigneaud, 1949).

The amino acid sequence of oxytocin was elucidated in 1950 (Pierce and Du Vigneaud, 1950) and the structure simultaneously by Du Vigneaud and coworkers and by Tuppy (Du Vigneaud and Trippett, 1953; Tuppy, 1953). The structure was proposed to be as follows:

H-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2

and this was confirmed with the total synthesis of oxytocin by Du Vigneaud in 1953 (Du Vigneaud et al, 1953). The chemical similarity between oxytocin and arginine vasopressin (found in man) foretold by Sharpey-Schafer was confirmed when the structure of arginine vasopressin was established in 1954 by Du Vigneaud's group (Du Vigneaud et al, 1954) to be the following:

Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2

The elucidation of the structures of oxytocin and arginine-vasopressin finally put to rest the dispute over the existence of one, two or more active principles contained in the posterior pituitary.

In tandem with these advances was the development of the concept of neurosecretion. The idea of axons serving as conduits through which chemical messengers were carried has its origins with Galen and Aristotle who interpreted the function of spinal nerves in this way. This understanding of how nerves function was widely accepted until it was finally challenged by the findings of electrophysiologists in the 19th century which were seemingly to the contrary. Experimental evidence to support the concept of neurosecretion was provided in 1919 when Carl Speidel published his observations on giant neurones in posterior spinal cord of elasmobranch fish which he described as possessing all the cytological requirements for secretory

function (Speidel, 1919). A similar finding was reported by Ernst Scharrer who identified neurones in the minnow brain which possessed the attributes of secretory cells upon histological examination (Scharrer, 1928). The term 'neurosecretion' was originally applied to the apparent function of any neurone which was found to contain particles similar to secretory granules normally associated with endocrine cells (Scharrer and Scarrer, 1940) but this was to change as understanding of neuronal structure and function improved. Posterior pituitary hormones were thought to be secreted by the pituicytes (Bucy, 1932) however, it was shown to be necessary to bilaterally ablate the supraoptic nuclei or to make lesions proximally in the neural stalk, causing atrophy of the posterior pituitary in order to produce diabetes insipidus (Fisher et al, 1938). In 1938 Rasmussen reported nerve fibres within the neural lobe and suggested that the neurohypophyseal hormones were released from these nerve terminals (Rasmussen, 1938). The full import of these observations was not appreciated until later reports utilising both hormone assay and histological analysis indicated a link between the activity of supraoptic and paraventricular nuclei and the posterior pituitary (Bargmann, 1949; Ortman, 1951; Hild and Zetler, 1953). Evidence continued to accumulate until Bargmann and Scharrer reported that transsection of the pituitary stalk resulted in accumulation of the neurosecretory substance proximal to the level of transsection and to a decline in the posterior pituitary content of hormone distally. There followed the proposal that the neurosecretory material was related to the neurohypophyseal hormones, was synthesised in the cell bodies of neurones in the supraoptic and paraventricular nuclei from where it was transported down the axons to the posterior pituitary where it accumulated to be secreted into the blood stream (Bargmann and Scharrer, 1951). This proposal revived the ancient concept of neurosecretion, at the same time restricting the application of the term according to new understanding of the neurosecretory process afforded most excellently by the hypothalamic supraoptic/paraventricular nuclei-posterior pituitary axis. The definition of a

neurosecretory system in mammals also fulfilled the promise of earlier work (Speidel, 1919; Scharrer, 1928) that neurosecretion might be demonstrated ubiquitously in a variety of species. As the means became available to distinguish between neurosecretory terminals, which generally contact basement membranes or glial elements in a non-synaptic way and normal neuronal terminals, classification of neurones as neurosecretory became less problematic (Bern and Knowles, 1966) and although branches of neurosecretory neurones may well terminate in conventional synapses, the quality unique to neurosecretory neurones is that of synthesising and then secreting a hormone in the traditional way described for non-neuronal endocrine cells.

Until the late 1950s the concept of neurosecretion rested mainly upon histological evidence, but the understanding of the neural control of hormone secretion was advanced significantly by the pioneering experiments of Barry Cross which aimed to define the neural control of oxytocin secretion from the neurohypophysis utilising the release of oxytocin from stimulated supraoptic and paraventricular neurones (Cross, 1966; Folley, 1969). Electrical stimulation of the paraventricular nucleus, the supraoptic nucleus or the supraoptico-hypophyseal tract resulted in effects upon the mammary gland and uterus of the rabbit which mimicked the injection of 10-50mU oxytocin and spinal transsection did not abolish this response (Cross, 1958a; Cross, 1958b). The possibility that such electrical stimulation of the hypothalamus might even initiate labour in the late-pregnant rabbit was tested and confirmed (Cross, 1958c). These experiments illustrated the striking correlation between electrical activity of neurosecretory neurones and hormone release. The neural control of oxytocin secretion was further defined by Cross and Green who recorded from putative magnocellular neurones whilst applying a hyperosmotic stimulus, known to liberate vasopressin (Cross and Green, 1959) and by a series of brain lesioning studies which mapped the central pathways of the 'Ferguson reflex' - the stimulation of oxytocin release in response to vaginal distension or dilation of the cervix (Cross,

1973).

In 1964, Kandel had succeeded in antidromically activating neurones in the preoptic nucleus of the goldfish thus enabling their identification (Kandel, 1964). In 1966, Yagi and coworkers extended this technique to the rat, using the ventral approach to the pituitary stalk (Yagi et al, 1966). They were followed by Richard Dyball who was able to antidromically activate neurones projecting from the paraventricular and supraoptic nuclei to the posterior pituitary by a transaural approach (Dyball, 1969). The technique of antidromic activation contributed to what was to be a major step forward in further defining the relationship between electrical activity in hypothalamic oxytocin- or vasopressin-secreting neurones (referred to henceforth as oxytocin or vasopressin neurones respectively) and has been described as, "...the most powerful tool available..." for their identification in vivo (Poulain and Wakerley, 1982). The use of this tool has made the hypothalamo-neurohypophyseal axis the best described example of a neurosecretory system.

Distinction between oxytocin and vasopressin neurones, both of which project to the posterior pituitary, and can be antidromically-activated became possible electrophysiologically in vivo by the way in which they responded to a variety of systemic stimuli designed to mimic physiological stimuli, thus allowing more precise study of the nature of the relationship between hormone release and electrical activity. In an early electrophysiological study Cross and Green (1959) had not found any difference between the shape of the action potential or pattern of electrical activity between hypothalamus, including supraoptic and paraventricular nuclei and other brain regions under basal recording conditions. However, the two neurohypophyseal hormones could be released to different degrees according to the stimulus used (Heller, 1966) suggesting the two hormones might be located in separate populations of neurones under differential control. More recent immunocytochemical experiments have demonstrated the separation of oxytocin and vasopressin into two magnocellular neuronal subgroups (Swaab et al, 1975; Vandesande and Dierickx, 1975) which are

not located separately in the two main magnocellular nuclei but are interspersed together. But given the difficulty in stimulating vasopressin release in isolation from that of oxytocin, the discovery that the suckling stimulus in the lactating animal selectively provokes the release of oxytocin was a landmark in the study of neurosecretion with particular application in the field of neurohypophyseal neurosecretion. Jonathon Wakerley combined antidromic activation of paraventricular oxytocin/vasopressin neurones with the application of a hungry litter to an anaesthetised lactating rat in order to follow both electrical activity and oxytocin release in terms of milk let down and the resultant increase in pup weight as they suckled. After some time throughout which the pups suckled continuously, the litter were observed to simultaneously stretch and suck more energetically for 10-15 seconds. This episode occurred repeatedly at 5-10min intervals. This was the first report of the milk-ejection reflex, which was shown to be the result of the intermittent release of a pulse of oxytocin (1-2ng) but not of vasopressin in response to a continuous suckling stimulus and which was preceded by a characteristic high frequency (50-80Hz) burst of activity in a proportion of antidromically-activated neurones (Wakerley and Lincoln, 1971a; Lincoln et al, 1973; Wakerley and Lincoln, 1973; Lincoln and Wakerley, 1974). Thus the electrophysiological differentiation of oxytocin and vasopressin neurones in vivo was possible based on their responses to the suckling stimulus (Poulain et al, 1977).

A stimulus usually associated with stimulation of the vasopressin system, systemic hyperosmotic challenge, in fact also excites oxytocin neurones and increases oxytocin secretion; the firing rate of putative oxytocin neurones is gradually increased in response (Brimble and Dyball, 1977; Blackburn et al, 1987). The mechanism by which oxytocin neurones are excited involves the region anterior and ventral to the third ventricle (AV3V region) which is composed of cirumventricular organs for which the blood-brain barrier is less exclusive. The AV3V region mediates a tonic excitatory drive to oxytocin neurones and seems to relay to oxytocin neurones any

changes in systemic osmolality (Russell et al, 1988). However, the magnocellular neurones themselves are osmosensitive: bathing of slices of hypothalamus containing supraoptic nucleus with a hyperosmotic medium causes depolarisation of the membrane intracellularly recorded magnocellular cells which still occurs in the absence of any synaptic input (Mason, 1980; Abe and Ogata, 1982; Andrew and Dudek, 1983; Mason et al, 1983; Andrew and Dudek, 1984; Bourque, 1989). The innate osmosensitivity of supraoptic magnocellular neurones is thought to be fully expressed only in the context of the full complement of normal synaptic inputs, including the AV3V region, which by their positive drive perhaps amplify the osmosensitivity already present (Dyball and Leng, 1990).

The identification of neurones was enabled further by the classification of electrical activity in the supraoptic and paraventricular nuclei into three categories: phasic (periods of activity interspersed with periods of silence, Wakerley and Lincoln, 1971b), continuous or slow-irregular activity whose parameters have been defined elsewhere (Wakerley et al, 1978). Later studies have confirmed that phasic activity is almost always associated with vasopressin-containing neurones in vivo (Yamashita et al, 1984) and in vitro (Yamashita et al, 1983; Cobbett et al, 1984) and whilst vasopressin neurones may be silent or may exhibit slow-irregular and continuous electrical activity, they are characterised by phasic activity when stimulated appropriately with an osmotic stimulus or by haemorrhage (Wakerley et al, 1975; Brimble and Dyball, 1977; Poulain et al, 1977). So, it is now possible with this foundation laid to identify neurones as oxytocin- or vasopressin-secreting with some confidence and thus to more accurately ascribe properties to one or the other cell type. These methods of identification of oxytocin neurones were made use of in all electrophysiological experiments which contribute to this Thesis such that conclusions could be drawn about the population of oxytocin neurones selectively.

I.2. Exogenous opiates. endogenous opioids and the regulation of oxytocin secretion

Although opiate effects upon vasopressin release were investigated with some

vigour (De Bodo, 1944; Duke et al, 1951; Giarman and Condouris, 1954; Schneiden and Blackmore, 1955; Inturrisi and Fujimoto, 1968) an interaction between morphine and oxytocin was not investigated until as late as 1978 when Haldar and Sawyer found that subcutaneous administration of morphine in lactating mice inhibited weight gain by suckling young (an index of milk ejection from the mammary gland). This was not the result of a loss of sensitivity of the mammary gland to normal levels of circulating oxytocin as exogenous oxytocin produced similar results in terms of pup weight gain indicating that mammary gland milk content in control and morphine-injected groups was the same. The conclusion was that morphine inhibited the release of oxytocin in response to the suckling stimulus in the lactating mouse (Haldar and Sawyer, 1978). This finding was later extended to the rat (Clarke et al, 1979). More broadly, oxytocin secretion stimulated by suckling, by angiotensin II and cholinergic drugs is inhibited by of exogenous opiates including morphine and opioid peptides (Haldar et al, 1982; Russell and Spears, 1984).

Endogenous opioids appear to inhibit the release of oxytocin from the neurohypophysis as revealed by naloxone (Bicknell and Leng, 1982). There is evidence that although endogenous opioids inhibit oxytocin secretion at the level of the neurohypophysis, morphine does not act there to inhibit release (Coombes and Russell, 1989) but that morphine can act at the cell bodies of these neurones to inhibit electrical activity. This is contrary to what was concluded from an earlier study in which intracerebroventricular morphine (2-4μg) failed to inhibit the bursting activity or background activity of identified oxytocin neurones during suckling (Clarke et al, 1979). Although opioids were later shown to indeed act at the posterior pituitary directly to inhibit oxytocin but not vasopressin release (Bicknell and Leng, 1982) the conclusion from the above study that opioids act at the level of the posterior pituitary but not at the perikarya to inhibit oxytocin release has been contradicted by in vitro experiments in which opioid peptides reduced the background electrical activity of some paraventricular neurones (Muhlethaler et al, 1980; Pittman et al, 1980) and of

putative oxytocin neurones in the supraoptic nucleus (Arnauld et al, 1983; Wakerley et al, 1983; Pumford et al, 1987; Inenaga et al, 1990). In addition still more recent work has shown that intracerebroventricular morphine does in fact inhibit the electrical activity of identified oxytocin neurones in the virgin female rat at doses lower (0.5-1μg i.c.v.) than those used by Clarke and coworkers (Leng and Russell, 1989). It is well established that there are opiate receptors in the posterior pituitary (Simantov and Snyder, 1977; Lightman et al, 1983a; Lightman et al, 1983b) which are predominantly or exclusively κ-opiate receptors (Bunn et al, 1985; Herkenham et al, 1986; Stojilkovic et al, 1987; Sumner et al, 1990). The receptors are functional, as the selective κ-opiate receptor ligand, U50,488H causes a dose-related inhibition of electrically-evoked oxytocin release which was naloxone-reversible (Zhao et al, 1988a). Although opiate receptors are present on pituicytes in the rat posterior pituitary (Lightman et al, 1983a; Lightman et al, 1983b), they have also been localised on the terminals of oxytocin/vasopressin-secreting neurones (neurosecretosomes) in the porcine and bovine posterior pituitary (Falke and Martin, 1985; Pesce et al, 1987) and these receptors have been shown to be functional in the rat posterior pituitary neurosecretosomal fraction in terms of inhibition of oxytocin release (Zhao et al, 1988b). In the same study, the μ-opiate receptor agonist, DAGO was inneffective in inhibition of oxytocin release. These data suggest that the inhibitory effect of morphine thought to occur at the posterior pituitary (Clarke et al, 1979) probably occurred by an action at the κ-opiate receptor subtype in the posterior pituitary, or that there was some inhibition of perikaryal electrical activity by morphine which was not evident from the neurones detected in the study.

Opiate effects at the cells bodies (Muhlethaler et al, 1980; Pittman et al, 1980; Arnauld et al, 1983; Wakerley et al, 1983; Leng and Russell, 1989; Inenaga, 1990; Leng et al, 1990) are explained by the presence of both μ- and κ-opiate receptors (Clark et al, 1986; Mansour et al, 1986; Tempel and Zukin, 1987; Mansour et al, 1988; Sumner et al, 1990; see also Chapter 5). Inputs to the supraoptic and

paraventricular nuclei are also possible sites of morphine action. The median preoptic nucleus and the subfornical organ, in, or projecting through the AV3V region respectively, provide a tonic excitatory drive to oxytocin neurones (Leng et al, 1988; Russell et al, 1988) and there are opioid receptors in the AV3V region (Mansour et al, 1987; Conrath and Cupo, 1989; Sharif and Hughes, 1989; Sumner et al, 1990; see Chapter 5) which contains morphine-sensitive cells (Buranarugsa and Hubbard, 1979). So, morphine and other opiates might act here to inhibit oxytocin release by reducing the electrical activity of the synthesising neurones. Still, when oxytocin neurones are tonically driven by stimulation of the AV3V region, morphine is still able to inhibit the electrical activity of SON oxytocin neurones (Leng and Russell, 1989). So a part of the acute inhibitory action of morphine is mediated by a direct action upon the cell bodies of oxytocin neurones.

I.3. Magnocellular oxytocin neurones: a neurosecretory system where tolerance to and dependence upon opiates develops

Although morphine given acutely by i.c.v. injection causes depression of oxytocin secretion and inhibits the electrical activity of oxytocin neurones (Russell, 1989), after 5 days of morphine given by i.c.v. infusion, oxytocin secretion is normal and in lactating rats the milk-ejection reflex returns to normal after 2 days (Bicknell et al, 1988a) indicating tolerance to an initial inhibitory action of i.c.v. morphine. This tolerance is not explained by enhanced production of oxytocin (Bicknell et al, 1988b) and neuronal content of oxytocin mRNA does not alter in morphine-tolerant rats (Sumner et al, 1989). The density of μ-type opioid receptors in the supraoptic nucleus is reduced after chronic morphine treatment and this might contribute to the observed tolerance (Sumner et al, 1990).

As well as tolerance, dependence develops to this regime of i.c.v. morphine (Bicknell et al, 1988a; Rayner et al, 1988; Leng et al, 1989b). This is revealed after the administration of naloxone which precipitates 'withdrawal excitation' rather than reversal of morphine inhibition. The firing rate of identified oxytocin neurones rises

to 350% control firing rate and oxytocin secretion increases by 80-fold (Bicknell et al, 1988a). There is no tolerance to or dependence upon morphine demonstrable at the posterior pituitary (Bicknell et al, 1985b) and morphine does not act here acutely to inhibit oxytocin secretion (Coombes and Russell, 1989) and therefore is unlikely to chronically. So, morphine tolerance and dependence are manifested at the cell bodies of oxytocin neurones where opiate receptors of the μ- and κ-subtypes exist (see earlier Introduction and Chapter 5). However, tolerance and dependence do not develop opiate receptors in the posterior pituitary, which, being primarily or exclusively of the κ-subtype (Bunn et al, 1985; Herkenham et al, 1986; Stojilkovic et al, 1987; Zhao et al, 1988b; Sumner et al, 1990) would bind morphine, a relatively selective agonist at the μ-type opiate receptor, poorly. The fact that a 350% increase in firing rate produced a 800% increase in oxytocin secretion is explained by the increased efficiency with which oxytocin is released as firing rate increases, frequency facilitation which has already been demonstrated for oxytocin neurones (Bicknell, 1988 and see Chapter 2). That the firing pattern elicited by naloxone after 5 days of i.c.v. morphine is sufficient to account for the observed disproportionately high secretion of oxytocin was confirmed by use of the recording of an oxytocin neurone withdrawing from chronic i.c.v. morphine after naloxone to stimulate the neurohypophyseal stalk in a morphine-naive control rat. The same profile of oxytocin release was obtained as was seen originally in the morphine-tolerant rat (Bicknell et al, 1988a).

This Thesis extends some of the above findings by the use of morphine and U50,488H to invesigate opiate effects upon the cell bodies of oxytocin neurones in this neurosecretory system. Because of the accessibility of these neurones as well as the relatively advanced state of current understanding of their function, the study of opiate effects in the supraoptic nucleus offers the opportunity to understand in greater depth the mechanisms of both acute and chronic opiate action on neurones. Given the presence of endogenous opioid peptides both centrally and peripherally in relation to

these neurones, the potential role that endogenous opioid peptides might play, should tolerance to and dependence upon them develop in a manner similar to that seen with morphine, must be a point of speculation.

Possible means by which oxytocin neurones adapt to chronic morphine exposure are investigated in this Thesis (see Chapters 3, 4 and 5) including the possible involvement of the AV3V region in the expression of dependence (see Chapter 6). Tolerance to i.c.v. morphine in terms of the electrical activity of oxytocin neurones is quantified (see Chapter 1). Whether tolerance to chronic i.c.v. morphine confers tolerance to U50,488H, the selective agonist acting at the κ-opiate receptor subtype acutely injected intravenously was also investigated (see Chapter 2).

CHAPTER 1

TOLERANCE TO MORPHINE IN OXYTOCIN NEUROSECRETORY NEURONES IN THE RAT: QUANTIFICATION OF TOLERANCE IN TERMS OF NEURONAL ELECTRICAL ACTIVITY

1.1. INTRODUCTION

Morphine inhibits the secretion of oxytocin (OT) (Grell et al, 1988) as illustrated by its effect on the milk ejection reflex (Haldar and Sawyer, 1978) and on parturition. Parturition is slowed by either peripheral or intracerebroventricular (i.c.v.) administration of morphine without an effect on the sensitivity of the uterus to OT and this effect is reversed by naloxone or OT administration (Russell et al,1989a). The milk-ejection reflex in the lactating rat is inhibited by acute i.v. morphine and this is not by an action at the mammary gland which remains sensitive to exogenous OT (Russell and Spears, 1984). Morphine was reported to act at the posterior pituitary in the lactating rat without any effect on the electrical activity of OT neurones in the supraoptic nucleus (SON), either on their background activity or on the high frequency bursting that precedes a milk-ejection (Clarke et al, 1979). The conclusion from this study was that morphine acts exclusively at the posterior pituitary to prevent OT release from neurosecretory terminals by the uncoupling of normal stimulus-secretion mechanisms. Although κ-opiates do act at the posterior pituitary to inhibit the release of OT by an action at the κ-receptor (Zhao et al, 1988b) known to exist in the posterior pituitary (Bunn et al, 1985; Herkenham et al, 1986; Sumner et al, 1990) there are few if any μ-type opiate receptors in the posterior pituitary (Bunn et al, 1985; Herkenham et al, 1986; Sumner et al, 1990) and there is no evidence for μ-receptor mediated inhibition of OT release upon stimulation of the pituitary stalk in vivo (Coombes and Russell, 1988). As morphine is a highly selective ligand at the μ-receptor with very low relative affinity for the κ-receptor (Kosterlitz, 1985) then it seems highly improbable that morphine does act at this site primarily to exert its undisputed inhibitory influence on OT secretion (Wakerley et al, 1983).

We determined to re-examine the effects of morphine on the cell bodies of OT-secreting neurones located in the SON.

Dependence develops to chronic morphine in terms of electrical activity of OT

neurones of the SON and this is expressed upon administration of the opiate antagonist, naloxone. Identified OT neurones respond with a large increase in firing rate and this is accompanied by a massive and sustained secretion of OT into the plasma. During the process of chronic morphine infusion according to our protocol, the development of tolerance is apparent. Tolerance is evident when after an initial dose of drug, subsequent doses must be increased in order to achieve the same effect as that of the first dose, that is, there is a loss of effectiveness of the drug with repeated administration (Jaffe, 1985). In the OT secreting system, tolerance is suggested by the resumption of the milk-ejection reflex in the post-parturient lactating rat and by normal plasma levels of OT in lactating rats (Bicknell et al, 1988a; Rayner et al, 1988) showing that morphine infused i.c.v. became less potent with time in these respects. Tolerance is incomplete however as a large enough intravenous dose of morphine was still inhibitory to the milk-ejection reflex in 5-day i.c.v. morphine-infused lactating rats (Rayner et al, 1988). Up to this point tolerance was inferred from behavioural and endocrinological indices.

In this study the primary aim was to confirm and quantify tolerance to i.v. morphine, in terms of electrical activity of identified OT neurones of the SON of rats treated chronically with i.c.v. morphine sulphate thus locating opiate effects as centrally-mediated. Second, confirmation was sought that dependence upon morphine develops within this protocol.

1.2. METHODS

1.2.1. Animals

Virgin female Sprague Dawley rats were used; housed at 21-23°C and allowed food (standard breeder diet) and tap water ad libitum; they were kept under a 13h/11h light/dark cycle.

1.2.2. Preparation for electrophysiology

1.2.2.1. Control group

Virgin female Sprague Dawley rats (mean body weight ± SE: 273 ± 5.8g, n=19) were anaesthetised with ether and placed into a stereotactic frame. The dorsal skull was exposed in the mid-line and 1.3mm holes drilled through the bone lateral to the cannula placement to take two stainless steel screws (3.2mm x 10 BA). A guide hole (1.0mm) was also drilled through which to introduce the infusion cannula. The infusion assembly was constructed as follows: the stainless steel cannula was made from a 1cm length of 21gauge tubing bent at 90° 4.5mm from its tip and bevelled at 45°. This was attached to a 18cm coiled length of polythene tubing (1.2mm o.d., 0.76mm i.d.) which was attached at the other end to a microsyringe. The whole assembly was positioned close to the skull and the stainless steel cannula inserted vertically into the right lateral cerebral ventricle (2mm right lateral, 3mm posterior to bregma according to the atlas of Konig and Klippel (1963)). Once in place, dental acrylic was applied to the surface of the skull, to cover the protruding cannula and the screws, there to provide anchorage, and it was left for several minutes to harden. An osmotic minipump (1μl/h, Alzet 2001, Alza Corp.) was placed subcutaneously in the subscapular region, the microsyringe detached and the cannula connected instead to the minipump. The infusion assembly was filled with sterile pyrogen-free water (vehicle).

1.2.2.2. Chronic i.c.v. morphine-treated group

Virgin female Sprague Dawley rats (mean body weight ± SE: 268 ± 6.8, n=17)

were anaesthetised with ether and cannulated intracerebroventricularly, as described above. Morphine sulphate B.P. was dissolved in sterile pyrogen-free water and filtered (Millex-GS, 0.22μm, Millipore S.A.). All dilutions were made from this stock solution which was kept at 4°C. The minipump was filled with a 50mg/ml solution of morphine sulphate and primed in 0.9% saline overnight at room temperature. The infusion cannula was filled with morphine sulphate solution in increasing concentration (10μg/h for 40h, 20μg/h for 40h and 50μg/h for 40h), separated by 1μl air bubbles in order to increase the degree of tolerance achieved over the 5 days.

1.2.3. Electrophysiology

On the sixth day of i.c.v. infusion rats were anaesthetised with urethane (ethyl carbamate, 1.25g/kg i.p.) and the trachea and a jugular vein cannulated. The rat was mounted in a stereotactic frame and the right SON and neurohypophyseal stalk were exposed surgically (ventral surgery performed by Dr G. Leng; Leng, 1981). A concentric bipolar stimulating electrode (SNEX 200, Rhodes Medical Instruments) was placed on the neurohypophyseal stalk and a glass micropipette filled with 0.9% saline (20-40 MOhm) was placed under visual control into the SON to record extracellularly from antidromically-activated neurones. Neurones were antidromically activated by stimulation of the neurohypophyseal stalk (1ms matched biphasic pulses <1mA peak to peak, 0.3Hz) and a measure of threshold voltage to activation made. Recorded neurones were tested for constant latency to antidromic activation and by the collision test to confirm their uninterrupted projection to the posterior pituitary. Thus neurosecretory neurones in the SON exclusively were identified and tested. The study was primarily concerned with putative OT-secreting neurones, so any neurones considered to be firing phasically (alternating periods of activity at >3Hz and quiescence for >5 seconds) which are putative vasopressin-secreting neurones (Poulain and Wakerley, 1982) were not studied beyond the initial control periods. Recordings of non-phasic neurones were made and stored on video tape in digital format via a modified pulse code modulator for later analysis. At the same time, spike

activity was used to trigger a digital pulse via a Unilab Interface. The time between pulses was measured with a BBC B+ microcomputer with software written for this application (Richard Bunting, Dept. Neuroendocrinology, A.F.R.C. Institute of Animal Physiology and Genetics Research, Babraham, Cambridge) and frequency histograms based on the number of pulses registered in 30 seconds (or an alternative bin duration) were generated. Firing rate was recorded for at least 10 minutes to establish a stable control period and morphine sulphate in 0.9% saline was injected via the venous cannnula in increasing dose: 1, 10, 50, 100, 500, 1000μg/kg up to a maximum dose of 5mg/kg in an injection volume of 500μl/kg. If a previously active neurone became silent for the 10 min following drug injection, or the upper limit of the dose range was reached, naloxone was administered (up to 5mg/kg i.v.). The mean firing rate for the 10 minute period corresponding to a particular drug was calculated and a significant change in firing rate was defined by the separation of the 95% confidence bands about the mean firing rate for control and test periods. Each test period mean firing rate was also expressed as a percentage of the control mean firing rate. Due to the lengthy protocol, units were occasionally 'lost' usually due to mechanical problems. In such instances, data was collected from another cell in the same rat and this was included only if the preceding dose had not affected the original cell and the next two doses of morphine were ineffective against the cell under test, suggesting that the threshold for inhibition was not reached.

1.2.4. Statistical analysis

The threshold effective dose of morphine to inhibition for the i.c.v. morphine and i.c.v. vehicle-treated groups were compared with the Wilcoxon test for independent samples and by the proportion of cells affected at each dose.

1.3. RESULTS

1.3.1. Characteristics of supraoptic non-phasic putative oxytocin neurosecretory neurones

1.3.1.1. Vehicle-infused rats

Control period recordings were taken from 59 cells in 17 i.c.v. vehicle-infused rats. 21 (35.6%) fired phasically and were classified as putatively vasopressinergic, 25 (42.4%) fired continuously at >2Hz and were classified as putatively oxytocinergic, 9 (15.3%) fired below 2Hz and irregularly and 4 (6.8%) were silent.

Mean ± SE latency to antidromic activation in active non-phasic cells was 13.7 ± 0.8 ms (n=24 cells; 17 rats) and the mean ± SE threshold voltage for antidromic activation was 27.8 ± 5.7 V (n=12 cells).

The mean ± SE basal firing rate of active non-phasic cells was 4.6 ± 0.6 Hz, n=30.

1.3.1.2. Morphine-infused rats

In 17 morphine-infused rats control period recordings were taken from 72 cells. 27 (37.5%) were phasic (not significantly different from the i.c.v. vehicle-treated group), 21 (29.2%) were continuously firing at > 2 Hz (not significantly different from the i.c.v. vehicle-treated group), 13 (18.1%) fired at less than 2 Hz (not significantly different from the i.c.v. vehicle-treated group) and 11 (15.3%) were silent (not significantly different from i.c.v. vehicle-treated group).

Mean ± SE latency to antidromic activation of non-phasic cells was 13.0 ± 1.0 ms (n=23 cells; 15 rats; not significantly different from the i.c.v. vehicle-treated group) and mean ± SE threshold voltage to antidromic activation was 38.1 ± 5.9 V (n=11 cells; not significantly different from the i.c.v. vehicle-treated group).

The mean ± SE basal firing rate of active non-phasic cells was 3.5 ± 0.7 Hz (n=28 cells in 17 rats; $P < 0.05$ vs. i.c.v. vehicle-treated group).

Silent and slow-irregularly firing neurones are identifiable as oxytocinergic or vasopressinergic only upon excitation when the firing pattern of one or other cell type

is adopted. Silent cells could not be tested anyway, because the inhibitory effects of morphine were under study. However, some cells from the slow-irregular category were tested in the i.c.v. morphine-treated group. The difference in mean firing rate of active non-phasic cells between groups was the result of the reduced firing rate of cells firing at less than 2Hz in the i.c.v. morphine-treated group.

1.3.2. Characteristics of supraoptic phasic putative vasopressin neurosecretory neurones

The mean ± SE basal firing rate in the i.c.v. vehicle-treated group was 4.2 ± 0.6 Hz (n=19 cells in 9 rats) and in the i.c.v. morphine-treated group, 6.4 ± 0.7 Hz (n=19 cells in 14 rats). The mean ± SE latency to antidromic activation was 14.6 ± 0.9 ms (n=12 cells) and 11.7 ± 0.5 ms (n=22 cells) in i.c.v. vehicle- and i.c.v. morphine-infused rats respectively ($P < 0.05$). The mean threshold voltage to antidromic activation was 27.8 ± 4.1 V and 26.7 ± 5.6 V (n=17 cells, not significantly different).

1.3.3. Effects of i.v. morphine on non-phasic putative oxytocin supraoptic neurones in i.c.v. vehicle-infused rats

Only inhibitory effects of i.v. morphine were seen.

The cumulative threshold dose of morphine needed to inhibit significantly the 6/7 continuously active neurones that responded was from 1μg/kg to 161μg/kg (median dose: 20μg/kg, 7 rats; see Figures. 1.3.3.1. and 1.3.5.2.). At a cumulative dose of 161μg/kg, all 6 neurones were markedly inhibited. The mean ± SE firing rate of these 6 neurones was 4.4 ± 1.1 Hz. The control firing rate of the neurone that did not respond to morphine was 14.0 Hz and was possibly an excited phasic neurone. The effect of morphine was evident within 30 seconds of i.v. injection of morphine. At the highest doses used, firing rate was reduced by a mean ± SE of 91.6 ± 3.3% (n=6 cells).

In those cells tested with naloxone HCl (5mg/kg), firing rate was restored to 97.7 ± 12.4% (n=4) of the control firing rate. There was no evidence of overshoot of the

firing rate to beyond that seen in the control period.

Figure 1.3.3.1.(overleaf) I.c.v. vehicle-infused group: summary of firing rate changes

Each histogram is the record of one neurone in one rat which had received an i.c.v. infusion of vehicle over the previous 5 days and was then urethane-anaesthetised for extracellular recording from identified neurones in the SON.

Firing rate of putative OT neurones is expressed as a percentage of the control firing rate measured in 30sec bins during a control period of at least 10minutes. The intravenous dose of morphine is given on the horizontal axis in μg/kg and ranges from 1μg/kg to 5000μg/kg at 10 minute intervals. Inhibition was defined as the firing rate during any 10 minute drug treatment period for which the 95% confidence bands about the mean did not overlap with those of the control period. Statistically significant inhibitions are marked with an asterisk. The control period firing rates were a) 9.3Hz b) 4.4Hz c) 2.3Hz d) 2.2Hz e) 3.8Hz f) 4.55Hz g) 14.0Hz. All cells except one (g) were significantly inhibited by 161μg/kg morphine or less.

Naloxone (NAL) where given fully reversed the acute inhibitory effects of morphine but did not result in an overshoot in activity.

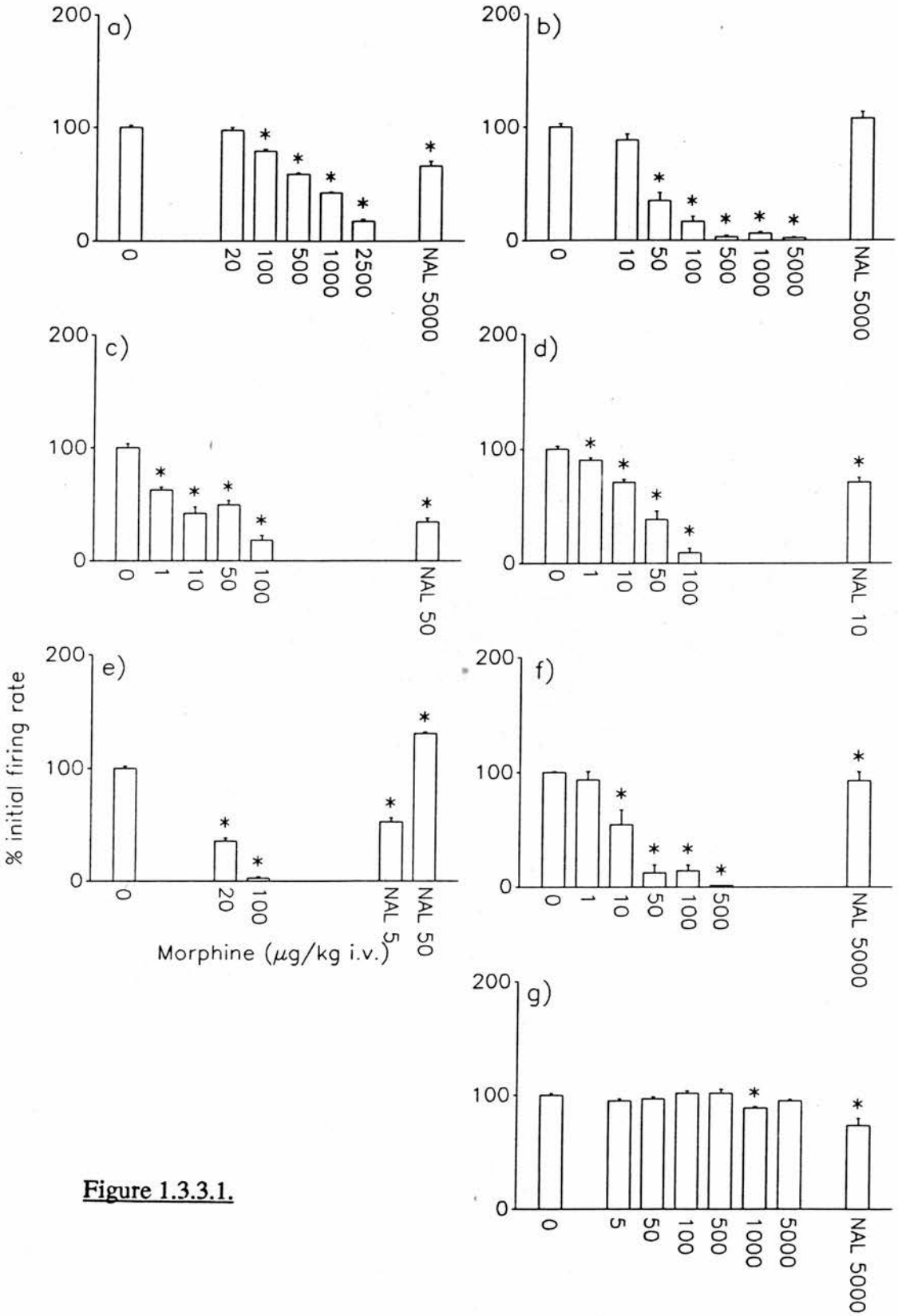

a)
b)
c)
d)
e)
f)
g)
200
100
0
0
20
100
500
1000
2500
NAL 5000
10
50
5000
1
NAL 50
NAL 10
5
% initial firing rate
Morphine (μg/kg i.v.)

Figure 1.3.3.1.

1.3.5. Effects of i.v. morphine on non-phasic putative oxytocin supraoptic neurones in i.c.v. morphine-infused rats

The mean ± SE basal firing rate of these neurones was 3.2 ± 1.2 Hz (n=9, 8 rats; not significantly different from the i.c.v. vehicle-treated group). Of this group, 5 neurones fired continuously at >2 Hz(mean ± SE firing rate 4.9 ± 1.8 Hz) and 4 were slow-irregular cells (mean ± SE firing rate 1.0 ± 0.15 Hz).

Only 4/9 non-phasic neurones were inhibited by morphine; the cells which did not respond received a cumulative dose of i.v. morphine of 161μg/kg (1 cell), 661μg/kg (2 cells), 6661μg/kg (1 cell) and 9110μg/kg (1 cell). The threshold cumulative dose of i.v. morphine to inhibition in those cells which were inhibited was 661μg/kg (1 cell), 1660μg/kg (1 cell), 6.600mg/kg (1 cell) and 6.661mg/kg (1 cell). The median threshold cumulative dose to inhibition was 1660μg/kg (n=9 cells; significantly greater than in the i.c.v. vehicle-treated group, P = 0.01). Whereas at a cumulative dose of <=161μg/kg 6/7 cells tested in the i.c.v. vehicle group were inhibited, none from the i.c.v. morphine-infused group were inhibited in a dose-dependent way (see Figures 1.3.5.1. and 1.3.5.2.).

At the highest dose of i.v. morphine used, 6.661mg/kg, the firing rate was reduced by 54.2% ± 10.8% (mean ± SE; n=5 cells, significantly different from the i.c.v. vehicle-infused group at lower doses, see above, P < 0.02).

At low doses of i.v. morphine excitatory effects were seen in 5/9 non-phasic cells (to 128.6 ± 8.9% control mean firing rate at 11, 60, 161, 500 and 661μg/kg). Of these cells however none remained excited at the threshold cumulative dose to inhibition for the morphine-infused group as a whole, 1660μg/kg if tested at that dose (3 cells). Two cells were lost at 161μg/kg and 661μg/kg and were excited by those doses, so reversal of the initial excitatory effect was not demonstrated.

Naloxone (5mg/kg i.v.) produced withdrawal excitation compared with the control mean firing rate. In the first 10 minutes after naloxone mean firing rate was increased to 260.4 ± 31.3% of mean control firing rate (P < 0.01, Student's paired t-test, n=4)

and this was significantly different from the response in the i.c.v. vehicle-infused group ($P < 0.01$, Student's unpaired t-test and Wilcoxon test for independent samples).

Figure 1.3.5.1.(overleaf) I.c.v. morphine-infused group: summary of firing rate changes

As in figure 1.3.3.1., each histogram is the record of a single neurone in one rat which in this case had received a 5-day i.c.v. infusion of morphine sulphate in increasing dose and was then anaesthetised with urethane. Intravenous morphine was given at 10 minute intervals and firing rate collected in 30 sec bins for calculation of firing rate over the 10minutes. A significant change was denoted by separation of the 95% confidence bands of the control firing rate and those of any drug treatment period. Only one neurone was inhibited by 161μg/kg morphine or less but this effect did not appear to be dose-related (cf. neurones from the i.c.v. vehicle-infused group). 5 cells were excited by low doses of morphine. Naloxone (NAL; 5mg/kg) produced hyperexcitation in all cells tested in this group. Control firing rates were a)3.1Hz b) 2.3Hz c) 3.6Hz d) 3.5Hz e) 12.1Hz f) 1.5Hz g) 0.85Hz h) 0.8Hz i) 1.0Hz. Cells a) - e) were classified as continuous putative OT neurones; neurone f) although slow-firing was classified as putatively oxytocinergic by its response to naloxone and neurones g) - i) cannot be classified definitely as putatively oxytocinergic or vasopressinergic.

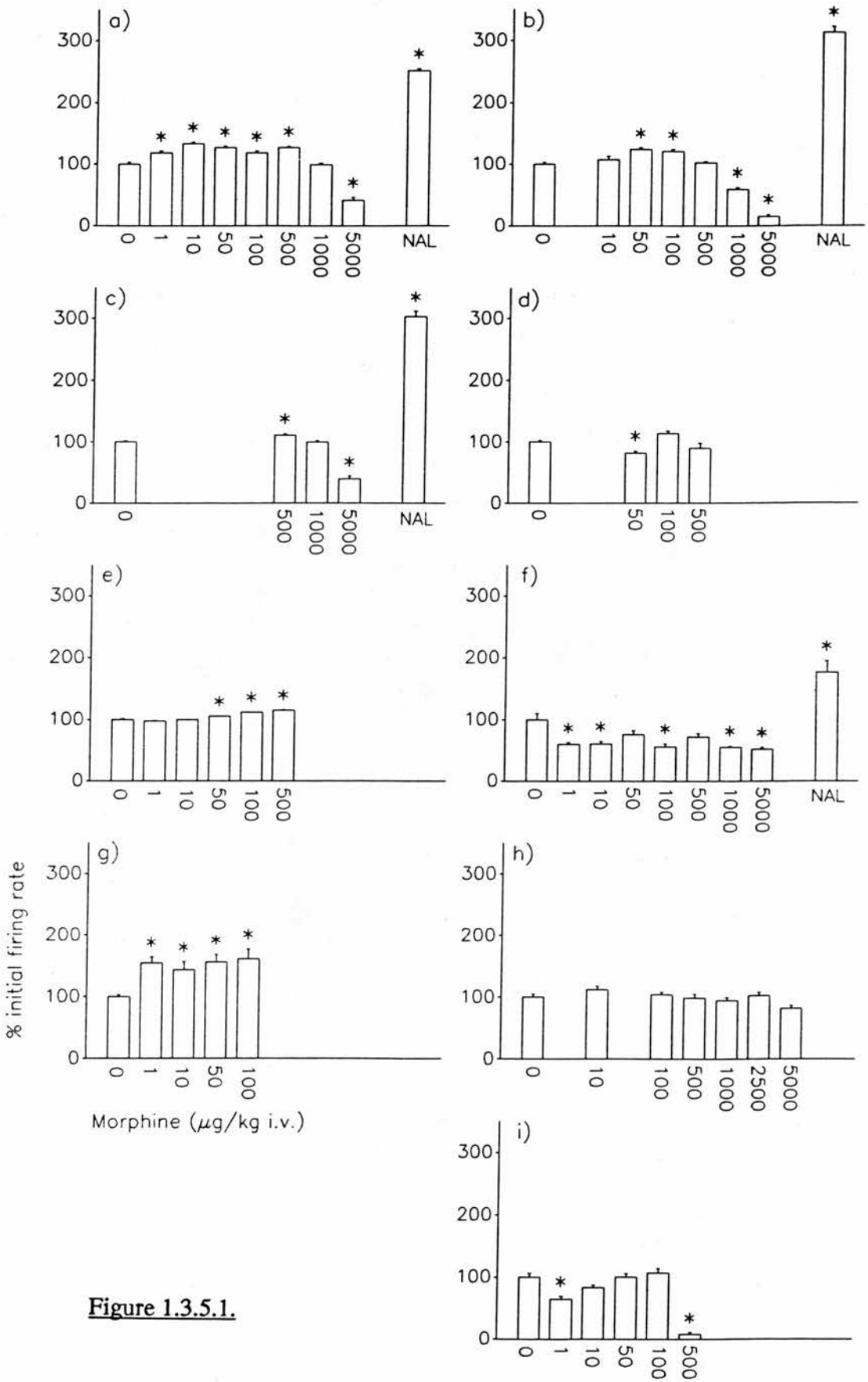


Figure 1.3.5.1.

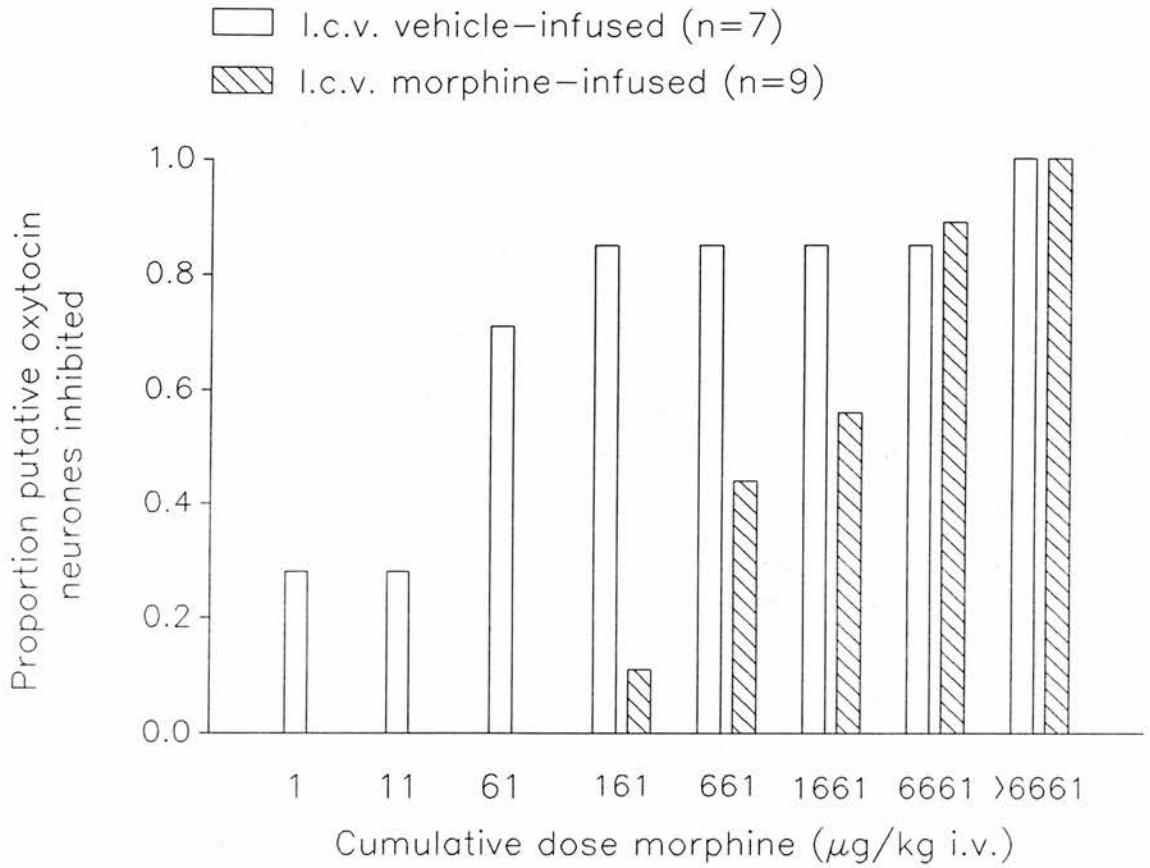


Figure 1.3.5.2. Comparative dose response relations for supraoptic putative oxytocin neurones from 5-day i.c.v. vehicle-infused and 5-day i.c.v. morphine-infused rats

Horizontal axis denotes cumulative dose of morphine (μg/kg i.v.) and the vertical axis, the proportion of neurones in each group which were inhibited by a given intravenous dose of morphine. At 161μg/kg morphine most of the neurones in the control group were inhibited whereas only one of the neurones from the 5-day morphine-infused group was.

1.4. DISCUSSION

1.4.1. Technical considerations

The protocol used lasted at least 80 minutes and longer if the naloxone response was followed beyond the initial 10 minutes. It was not always possible to complete the protocol on one cell. If a cell responded maximally to morphine before the end of the full dose range was reached, then no further dose of morphine was injected and the inhibitory response was instead challenged with naloxone. Recordings were occasionally abandoned part way through if it became apparent that the cell under study was a phasic, putatively vasopressinergic cell and therefore of no further interest in this particular study. Some recordings were 'lost' as a result of sudden mechanical damage to the cell. The region of the SON is highly vascularised (Akmayev, 1971). The surgical approach requires that the dura be incised discretely over a small part of the nucleus to allow passage of the pipette into the body of the nucleus. The stability of recordings seemed to be partly compromised by the degree of exposure of the tissue and this was always minimal. However, sudden movement of the pipette tip, which is very flexible laterally and may be close to pulsating capillaries, may account for the physical damage to neurones which suddenly emitted an injury discharge. Other cells were characterised by an extracellular spike which diminished in height gradually throughout the protocol in a way that appeared to be independent of drug treatment. This seemed to be a result of subtle movement of the cell in relation to the pipette, as advancing the pipette towards the cell could restore the quality of the recording. However, once in the nucleus, it was impossible to move the pipette in more than the one plane and this means that if a cell was moving away from the pipette laterally then no amount of advancing or retracking could restore the recording. In such cases, the protocol was abandoned as the signal-to-noise ratio became so small that reliable discrimination of the spike was no longer possible.

Some movement was probably due to tissue swelling that arose with repeated

penetration of the nucleus. Searches were confined to one or two tracks through the nucleus to minimise tissue swelling. This is not only advantageous in terms of making stable recordings, it also improves the chances of recording from physiologically "near normal" cells.

With the first cell lost, a replacement cell in the same nucleus was sought and the protocol continued, but not restarted, as it was a cumulative regime and the time lapse between 'losing' a recording and finding a replacement cell was usually too short. The plasma half-life of morphine in the rat is 1-2 hours for the fast phase and 10 hours for the slower phase (Berkowitz et al, 1974), so administering morphine every 10 minutes in increasing dose would result in its accumulation in the plasma: hence the expression of dose as cumulative rather than discrete.

In order to maximise the usefulness of these part records, a procedure was determined to select out records from which data could be legitimately extracted for mean control firing rate, median threshold effective dose and degree of reversal with naloxone, even after the administration of preceding doses. If a rat was exposed to up to 661μg/kg morphine or more (chosen retrospectively as being the likely threshold effective dose of morphine to inhibit these cells) and the cell lost then any subsequent recording did not contribute to calculation of control firing rate, threshold effective dose, or reversal of morphine induced inhibition with naloxone. If the last dose was less than 661μg/kg, and the next dose injected inhibited the new cell, no measure of threshold dose to inhibition was obtained. Should the first dose of the resumed regime not have inhibited the new cell, but a higher dose did and a dose related trend was apparent, then it was judged that the threshold dose to inhibition had not been reached for the new cell and it was then estimated.

For 1 cell in the control group, (Figure 1.3.3.1.b) the rat received 1μg/kg morphine more than 15 minutes before the 'control period' for this cell was recorded and the cell only later responded to i.v. morphine at 50μg/kg with a significant inhibition; for this cell then the 'control rate' was that measured long after the original lowest dose of

morphine. The mean control firing rate of the remaining 6 cells was 6.0 ± 1.9 Hz and the median firing rate 4.2 Hz. The mean firing rate is relatively high because of one fast-firing cell which had a control firing rate of 14.0 Hz and this cell did not respond to morphine. The control firing rate of cell b) (see Figure 1.3.3.1.), was 4.4 Hz.

In the chronic i.c.v. morphine-treated group, the control periods of 3/9 cells were after lowest doses of morphine: cells d), c) and b) (see Figure 1.3.5.1.): Cell d) (see Figure 1.3.5.1.) this rat received up to 11μg/kg more than 20 minutes before the tested cell was recorded. Although at 50μg/kg there was a 'significant' inhibition of firing rate, there was no dose related inhibition and a cumulative dose of 661μg/kg was not inhibitory compared to the 10 minute 'control period' for this cell. This suggests that the threshold inhibitory dose was not reached within the schedule and certainly not prior to the 'control period'. Cell c) (see Figure 1.3.5.1.): this rat received a cumulative dose of 161μg/kg 8 minutes before the commencement of the 'control period' recording for this cell. None of the other cells in this group were inhibited at this dose. The next dose, 500μg/kg was not inhibitory but excitatory and it was not until 5mg/kg (6500μg/kg cumulative dose) that this cell was inhibited. Clearly there was no evidence of the threshold inhibitory dose having been reached before the 5mg/kg dose at the end of the protocol. Cell b) (see Figure 1.3.5.1.): this rat received a single dose of 1μg/kg morphine i.v. 15 minutes before the start of the 'control period' for this cell. No inhibition was evident until a cumulative dose of 1660μg/kg was reached. Indeed, this cell was excited by cumulative doses of 60μg/kg and 160μg/kg, confirming that the threshold inhibitory dose probably was not 1μg/kg. The mean firing rate of the remaining 6 cells was 3.2 ± 1.8 Hz and the median firing rate 1.2 Hz. The control firing rates for the three above mentioned cells were 3.5, 3.6 and 2.3 Hz respectively. These values are well within the expected range for control cells from this group. From these cells therefore, data for control firing rate, threshold inhibitory dose and degree of naloxone reversal were obtained.

1.4.2. Effects of i.v. morphine on putative oxytocin cells from vehicle-infused rats

Acute i.v. morphine given to control (vehicle-infused) virgin female rats inhibited the spontaneous activity of antidromically-identified continuously-firing (putative oxytocin (OT)-secreting) neurones of the SON. These cells were exquisitely sensitive to morphine which was effective at a threshold dose of 60 μg/kg (n=5/6 cells affected; 1 cell: 120μg/kg; 1 cell unaffected). This dose of morphine is much lower than that required to inhibit the milk-ejection reflex, which reflects OT release in response to suckling (Russell and Spears, 1984; Clarke and Wright, 1984) but perhaps this is not surprising since low-dose electrophysiological effects are very short lived and are unlikely therefore to discernably affect the milk ejection reflex. Since morphine does not inhibit the release of OT evoked by stimulation of the pituitary stalk in lactating rats using a similar surgical technique and protocol (up to 5mg/kg; Coombes and Russell, 1988), and opiate inhibition of OT release at the level of the posterior pituitary is via the κ-type opiate receptor (Bicknell et al, 1985a) it follows that the primary, if not exclusive action of morphine in these experiments is at the cell bodies of oxytocinergic neurosecretory neurones in the SON or upon afferents to the nucleus. This concept is contrary to that of an earlier report (Clarke et al, 1979), which suggested that opiates act exclusively at the terminals of these neurones in the posterior pituitary to impair normal stimulus-secretion coupling, but is consistent with later experiments in the hypothalamic slice (Wakerley et al, 1983) which showed inhibitory effects of morphine upon putative OT neurones of the SON. Perhaps then the powerful stimulation to OT neurones upon activation of the milk-ejection reflex is too robust to be influenced by these transient electrical events occurring in the SON.

Morphine is a highly selective ligand for the μ-type opiate receptor with negligible affinity for the δ- or κ-type opiate receptor (Kosterlitz, 1985). At the low doses used in the control group of rats, it is therefore most likely to be acting at a μ-type receptor rather than at the κ-type, both of which we have found in the SON (Sumner et al, 1990; see Chapter 5) contrary to another report (Mansour et al, 1988).

In confirmation of this assumption, naloxone, which is an opiate antagonist with a 10-15 fold preference for the μ- rather than the κ- or δ-opiate receptors (Kosterlitz, 1985; Leslie, 1987) was effective at antagonising profound inhibition by doses of morphine up to 6661μg/kg. Naloxone was effective even at 100μg/kg in one cell (see Figure 1.3.3.1.d), which had been inhibited to 9.2% control firing rate by a cumulative dose of 161μg/kg morphine i.v.. Naloxone reversed this inhibition and restored the firing rate to 98.7% control firing rate. In another cell (see Figure 1.3.3.1.c), a lower dose still of naloxone was used to partially reverse the morphine induced reduction in firing rate: after inhibition to 18.4% control firing rate by a dose of morphine of 161μg/kg i.v., naloxone (50μg/kg) restored activity to 34.3% control firing rate. Unfortunately, higher doses of naloxone could not be tested in this cell as the recording was susequently lost. These two examples illustrate the sensitivity of neurones recorded in control rats to low doses of naloxone, consistent with an action through a μ-type opiate receptor to antagonise morphine-induced inhibition of these neurones at the level of the cell bodies or at afferent inputs to the SON, such as the SFO which also contains opioid receptors (Mansour et al 1987; Conrath and Cupo, 1989; Sharif and Hughes, 1989).

This result is corroborated by in vitro studies using the coronal hypothalamic slice containing SON (Pittman et al, 1980; Wakerley et al, 1983; Pumford et al, 1987). The cell bodies of putatively oxytocinergic neurones identified by firing pattern were inhibited by bath-applied opiates in a naloxone reversible way. Although some structural integrity remains in this preparation, afferents coursing over long distances in a different plane are transected and this mainly leaves a local afferent input. The site of action of opiates in the hypothalamic slice preparation and of i.c.v. morphine in the present experiments is not clear. It is certainly however a central site.

In most cells, it was thought more prudent not to try to demonstrate the receptor subtype likely to mediate the morphine inhibition, by testing low doses of naloxone as discussed above as this is not best investigated by this approach anyway. What was

more telling was the fact that maximal doses of naloxone did not evoke electrical activity beyond that measured during the control period for that cell i.e. there was no evidence of acute dependence. In cells from control rats tested with naloxone, the mean firing rate was 97.7 ± 12.4% ± SEM control firing rate (n=4). This was not due to an inadequate dose of naloxone. The dose used, 5mg/kg, is a large dose, widely used which remains selective for opiate receptors. Naloxone therefore was simply antagonising the accumulated circulating concentration of i.v. morphine in the control rats. So, within the time course of this protocol, acute dependence upon morphine did not develop. Additionally, it appears that there is no endogenous opioid inhibitory tone regulating these neurones as naloxone did not cause an overshoot in electrical activity. This contrasts with the inhibitory κ-opioid tone demonstrated in the posterior pituitary which results from dynorphin co-release with vasopressin which then acts upon opiate receptors on OT cell terminals to inhibit release (Zhao et al, 1988b). Figure 1.4.2.1. was constructed from data drawn from the present series of experiments alone. Data from the present series of experiments were combined with those from the series of experiments described in Chapter 2 (where neurones were tested with acute i.v. U50,488H instead of acute i.v. morphine) in order to increase group size and make trends more apparent and these data were used to construct Figure 1.4.2.2.. The results from this second series of experiments are fully described in Chapter 2. It was thought acceptable to include control data as U50,488H effects would not need to be considered and to include data on naloxone reversal and withdrawal as the responses of neurones after U50,488H were similar to those in the present series. So, in the vehicle-infused group, naloxone caused a reversal of opiate (morphine or U50,488H) inhibitory effects to 90.3 ± 14.8% control firing rate and in the 5-day morphine-infused group, to 337.4 ± 138.75% control firing rate. It appears therefore that the effects of U50,488H are also readily reversed by naloxone in the protocol and its inclusion did not impair the ability of naloxone to reverse acute effects of opiate nor its ability to precipitate a full withdrawal response to chronic i.c.v.

morphine. Considering then the overall firing rate of continuously-firing neurones in the SON after naloxone, as depicted in Figure 1.4.2.2.a): although the control firing rate in the absence of naloxone was similar in the vehicle-infused and the morphine-infused groups, there was a marked increase in firing rate of putative OT neurones in the morphine-infused group after naloxone (P=0.027, Wilcoxon, significantly different from the control group). This indicates that the supraoptic nuclear firing rate is raised significantly by administration of naloxone; it is not a response confined to a small number of neurones and is confirmation that this change in activity probably accounts for the increase in plasma OT already reported elsewhere (Rayner et al, 1988).

Acute tolerance to opiates has been reported in vitro (Gahwiler, 1981). Had tolerance developed during the i.v. administration of morphine to control rats, a trend in response unrelated to dose might have been expected. In fact, the response to i.v. morphine followed a predictable pattern of ever increasing magnitude of inhibition of firing rate as the dose administered was increased (see Figure 1.3.3.1.). The onset of tolerance does not seem to occur for some time in chronic i.c.v. morphine-treated rats (Rayner et al, 1988). It is several days before a rat receiving a chronic i.c.v. infusion of morphine solution in increasing dose seems to recover from the initially sedative action of morphine, the disruption of normal behaviour patterns and core body temperature elevation and the normal operation of the milk-ejection reflex, mediated by OT secretion. So, although the time course of the development of tolerance to and dependence upon morphine was not studied by interrupting this 5 day procedure at intervals to make assessment, it does not exist noticably until day 2-3 of i.c.v. infusion. This observation agrees with the absence of acute tolerance described above.

1.4.4. Effects of i.v. morphine on putative oxytocin cells from chronic i.c.v. morphine-infused rats

Although successive increasing doses of morphine were increasingly effective once the threshold dose of morphine was given, as in the control group, the threshold dose

of morphine was markedly different, indicating tolerance to i.v. morphine in these cells or their inputs after chronic i.c.v. morphine infusion. Control cells responded with a median threshold effective inhibitory dose of 20μg/kg morphine. At the median cumulative dose of 161μg/kg 6/7 control neurones were significantly inhibited. Chronic i.c.v. morphine treated cells however, were inhibited by morphine at the higher cumulative threshold dose of 1660μg/kg. At the median cumulative dose of 161μg/kg only 1 cell from this group was inhibited but not in a dose-related way. On this basis, the two groups were quite different (P=0.01, Wilcoxon).

There were excitatory effects of morphine at low doses which were succeeded by inhibitory effects at higher doses, where tested, in the morphine-infused group. These excitatory events were not seen in neurones from the vehicle-infused group (but see Chapter 3). The observation of opiate-induced excitation is not unprecedented. In cultured dorsal root ganglion neurones, opiates cause a prolongation of the action potential at nanomolar doses whilst shortening the action potential at micromolar doses in the same cell. This direct effect is achieved by modulation of a voltage-sensitive potassium or calcium channel (μ- or κ-ligand respectively) first one way, by low doses and then in the opposite way by higher doses of the same opiate (Crain and Shen, 1990). By extrapolation, shortening of the action potential would reduce voltage-dependent calcium entry into the terminals of neurones thus affected, impairing calcium-dependent neurotransmitter release; inhibition of neurotransnitter release is typically associated with opiates. Conversely, where opiates at low doses prolong the action potential, the same argument implies that opiates might directly facilitate neurotransmitter release. As well as the direct excitatory effects described above, opiates do have indirect excitatory effects in hippocampal slices for example, which involve interaction with inhibitory GABAergic synapses (Bradley and Brookes, 1984). So both direct and indirect excitation are possible. In dorsal root ganglion explant chronically exposed to 1μM DADLE, the acute action potential-shortening properties of the opiate (at 10μM) were diminished in most neurones whereas the

prolongation of the action potential after DADLE was seen more often (Crain et al, 1988). This implicates these excitatory events in tolerance as well as the more well documented inhibitory events in this system. As these excitatory events were not seen in the vehicle-infused group this perhaps suggests that the expression of this response to opiates has developed with tolerance and might be a marker of tolerance in supraoptic neurones. However, group sizes are perhaps too small to draw this conclusion firmly. More experiments with vehicle-infused rats might confirm or refute this. In particular, it would be necessary to explore the occurrance of the excitatory response at much lower doses of morphine in the vehicle-infused group. If the inhibitory response to morphine undergoes adaptive change, so might the excitatory response to morphine and so as the threshold cumulative dose to inhibition in the vehicle-infused group is much lower than that in the morphine-infused group, then perhaps so is the threshold excitatory response. It might therefore be demonstrated frequently at a lower dose range starting at 1ng/kg. As low dose excitation was not anticipated when the experiments were designed and as the primary aim of the series was to define the difference in the threshold dose to inhibition for morphine, then the phenomenon was not investigated further. So, whether the excitatory events are also affected by chronic opiate exposure awaits further study.

This prolongation of the action potential in dorsal root ganglion cells is meaningful in terms of neurotransmitter release. Enhancement of the capsaicin-evoked release of substance-P from primary afferent fibres by μ-opioid receptor agonists in rat spinal cord has been described (Pohl et al, 1989) and Sweeney and co-workers have demonstrated naltrexone-reversible opiate enhancement of endogenous adenosine from rat spinal cord synaptosomes in vitro (Sweeney et al, 1989). The biphasic response of some supraoptic neurones in this study was likewise seen in enteric ganglia where electrically-evoked enkephalin release is bimodal in response to μ-, δ- and κ-opiate receptor selective ligands. It is conceivable therefore that the OT neurosecretory system might respond to these excitatory low doses of morphine with a

measurable increase in the plasma concentration of OT. Whether this response occurs in response to endogenous opiates under certain physiological conditions is not known. The excitatory and inhibitory effects of the κ-opiate receptor agonist U50,488H both on firing rate of putative OT neurones and on plasma OT are considered in a later chapter (see Chapter 2).

In terms of basal electrical activity, the firing rate of non-phasic neurones from the control group was 4.6 ± 0.6 Hz (mean ± SEM) and from the i.c.v. morphine-treated group 3.5 ± 0.7 Hz ($P<0.05$, Wilcoxon). This value was constructed from continuous and slow-irregular neurones. Phasic (vasopressin) cells had similar control firing rates between the i.c.v. vehicle- and i.c.v. morphine-infused groups.

The spectrum of activity seen in 5-day vehicle-infused and 5-day morphine-infused groups was compared to see if the proportions of the four types of activity differ as a result of chronic i.c.v. morphine treatment (Figure 1.4.4.1.). After i.c.v. morphine treatment, the proportion of continuous neurones decreased and the proportion of silent neurones increased, though neither significantly so, reflecting a trend in the activity profile downwards. Whether this trend reflects a real change which would have achieved statistical significance in a larger sample of cells is not clear. The same trend was not seen in phasic (vasopressin) cells which were recorded in similar numbers between groups (Figure 1.4.4.1.). Because there is no difference in the proportion of cell types between groups, it appears that tolerance to i.v. morphine in OT cells of the chronic i.c.v. morphine-treated group is complete.

We have recently reported no difference in plasma OT concentration in conscious rats treated chronically with i.c.v. morphine as compared with untreated rats (Sumner et al, 1990); other reports indicate a slight depression after urethane anaesthesia in plasma OT concentration after the 5 day i.c.v. morphine infusion (Bicknell et al, 1988a; Sumner et al, 1989). Normal secretion of OT in chronic i.c.v. morphine-infused rats is not the result of increased synthesis: the OT mRNA content of these neurones is unchanged (Sumner et al, 1989) also, OT content in the magnocellular

nuclei and posterior pituitary in the lactating rat after 5 days of i.c.v. morphine is unchanged from control. OT release is not enhanced: the proportion of total OT content in isolated posterior pituitary released upon electrical stimulation is not altered (Bicknell et al, 1985b). Additionally, there were no differences in antidromic latency or threshold to activation for continuous neurones, suggesting that changes in axon or axo-somatic properties of conduction are not involved in adaptive changes with chronic morphine. These results suggest therefore that decreased effectiveness of i.v. morphine in the i.c.v. morphine-treated group at inhibition of supraoptic OT neurone elecrical activity could account for tolerance in the OT neurosecretory system. The dose response relation shifted to the right after chronic i.c.v. morphine reflecting that higher doses of morphine were needed to achieve the same inhibitory effect as the range of doses in the control group (Jaffe. 1985; see Figure 1.3.5.2.). A shift to the right has been reported for locus coeruleus neurones in vitro which become tolerant to morphine (North, 1988).

Interestingly the maximum dose of morphine used in the chronic i.c.v. morphine-treated group only depressed firing rate by a mean of 54% (n=5; 6661μg/kg and 9110μg/kg in one cell). One possible explanation of this phenomenon is that there are changes in the receptor/opiate interaction such that the full agonist response of near 100% inhibition of electrical activity in control cells (produced by occupancy of a small percentage of available receptors) is replaced with a partial agonist response which could never, even given maximal occupancy of receptors, stimulate a full response. Thus a 100% inhibitory response would not be attainable via receptors exposed chronically to opiate. This would result in a levelling off of the dose response relation prematurely. This switch from full to partial agonist by i.v. morphine would be explained by a change in the receptor conformation or post-receptor mechanisms. The drug would be said to be of lower efficacy (the 'strength' of a drug-receptor complex to evoke a response; Stephenson, 1956). In order to test this hypothesis, an extended range of doses of morphine could be used in a series of similar experiments.

Whether the shortfall in effect could be accounted for by insufficient opiate to occupy 'spare' receptors (Stephenson, 1956) or whether in fact the receptor pool is fully occupied and the response still not 'maximal' could be assessed. However, as I was also keen to demonstrate the naloxone-induced hyperexcitability of chronic i.c.v. morphine treated neurones, to highlight the development of dependence, the administration of very high doses of morphine to define the maximally effective dose might have impaired the naloxone response, thus calling into question the existence of dependence. So, this was not pursued in the present study.

An alternative explanation for the reduced level of 'maximal' inhibition in the chronic i.c.v. morphine-treated group could be that chronic morphine exposure enhances the capacity of the liver to metabolise circulating morphine, so that much higher doses of morphine are required to achieve the same bioavailability. Indeed methadone induces liver enzymes to enhance its own metabolism (Anggard et al, 1975). This is an unlikely scenario as it has been shown in rats that the development of tolerance does not alter the plasma half-life of morphine in rats (1-2 hours for the fast phase and 10 hours for the slower phase) and morphine can be detected in the plasma 20 days after removal of morphine pellets, so complete elimination is a slow process (Berkowitz et al, 1974). Morphine is mainly inactivated by conjugation to form morphine glucuronide, which is excreted in the bile and by the kidneys. Some hydrolysis of the glucuronide occurs in the gut with subsequent reabsorption. This enterohepatic circulation can cause the persistence of morphine.

The adaptive changes at the receptor or in post-receptor mechanisms that constitute tolerance could be explained in several ways. Neurosecretory neurones or their inputs may lose functional opiate receptors; existing opiate receptors may have a decreased affinity for the ligand thus necessitating a larger dose of opiate to achieve the same effect; coupling of receptors to intracellular mechanisms might be impaired, reducing the effectiveness of the ligand-receptor interaction, or an endogenous opiate antagonist may be produced which will compete with circulating opiate for receptor occupation.

In a recent study, we have reported by quantitative receptor autoradiography a selective down-regulation of the μ-type opiate receptor in the SON of 5-day i.c.v. morphine-treated rats (Sumner et al, 1990; see Chapter 5). This might contribute to the observed tolerance but the importance of the reduction in size of the pool of 'spare' receptors (Stephenson, 1956) and the relative importance of affinity changes must be considered. I have not investigated these aspects. As I was able to substantially inhibit neurones from chronic i.c.v. morphine-treated rats with high doses of morphine, this suggests that there is a pool of functional receptors similar in size to that in the control rat such that a near maximal response could be elicited. As mentioned earlier, the use of much higher doses of morphine in a similar series of experiments would indicate the true size of the pool of functional receptors.

Abruptly precipitated withdrawal from chronic morphine by naloxone in this group of rats produced a dramatic increase in firing rate which contrasted sharply with the control group response ($P<0.01$, Student's t-test and Wilcoxon). This appears not to be the just the reversal of accumulated intravenously-administered morphine. In the 10 minutes following naloxone injection, mean firing rate was increased to 260.4 $\pm$ 31.3% of mean control firing rate ($P<0.01$, Student's paired t-test, $n=4$). This 'withdrawal excitation' has been shown to adequately account for the observed hypersecretion of OT in the lactating rat on naloxone-precipitated withdrawal of chronic morphine (Bicknell et al, 1988a) and these results suggest that not only has tolerance developed within this schedule but dependence as well.

Part of this withdrawal excitation is mediated at a site or sites outside the blood-brain barrier (see Chapter 6). This is the conclusion from a study using a naloxone salt, naloxone methyl bromide, which does not cross the blood-brain barrier when given i.v. but which precipitated a partial withdrawal response in OT neurones after 5 days of i.c.v. morphine treatment (Bicknell et al, 1987a; Russell et al, 1989b). Morphine could be acting at several sites to acutely inhibit the activity of OT neurones, apart from the neurones themselves or local inputs. The subfornical organ

(SFO) and the region anterior and ventral to the third ventricle (AV3V region) contain opiate receptors (Mansour et al, 1987; Conrath and Cupo, 1989; Sharif and Hughes, 1989) and project to supraoptic OT neurones providing a tonic excitatory input (Russell et al, 1988; Leng et al, 1989a). However, tonic activity resulting from stimulation of this excitatory input can be inhibited by i.v. morphine (Leng and Russell, 1989) suggesting some modulation more locally to the neurosecretory neurones. Indeed, in vitro experiments in the rat hypothalamic slice containing SON have suggested opiate inhibition of OT cells occurs in or close to the nucleus (Pittman et al, 1980; Wakerley et al, 1983; Pumford et al, 1987). Wherever the opiate receptors are, they must be of sufficient number and have access to post-receptor mechanisms because withdrawal elicits massive activation of receptors, evidenced by the increase in electrical activity of OT neurones. The mechanisms of tolerance and dependence are not necessarily related and an explanation for either need not encompass the other (Wuster et al, 1985). It has been suggested that tolerance might be the result of an uncoupling of the receptor from post-receptor mechanisms thus reducing the pool of available functioning receptors (Wuster and Costa, 1984) and that dependence on the other hand might follow hypertrophy of intracellular mechanisms in response to continuing inhibition as a counteractive measure and that this requires an intact neuronal system and enough functioning receptors to mediate this inhibition, as when withdrawal is precipitated, the full response in reverse, and more is expressed through functioning receptors. Whilst I always saw tolerance together with dependence in this study, it is difficult to explain tolerance and dependence in terms of a unitary response and they have been shown to be dissociated in other systems (Christie et al, 1987). The experiments described in later Chapters looking at changes in receptor density and the involvement of G-proteins in mediating the acute effects of morphine in these neurones shed more light on possible mechanisms which undergo adaptive change in response to chronic opiate.

Figure 1.4.2.1.a) Firing rate (Hz) of putative oxytocin neurones from 5-day i.c.v. vehicle-infused and 5-day i.c.v. morphine-infused rats

Data were drawn from the two series of experiments described in Chapters 1 and 2 respectively.

The first point illustrated by this histogram is that initial firing rate is not different between the i.c.v. vehicle-infused and the i.c.v. morphine-infused groups (4.7Hz and 2.6Hz respectively; n.s., unpaired two-tailed t-test). To make the comparison, neurones from which a record of control firing rate had been obtained in the vehicle-infused groups from Chapters 1 and 2 contributed to a mean value for initial firing rate, hence the large group size (n=30). However, of neurones tested in the i.c.v. morphine-infused group, only neurones which received naloxone (5mg/kg) after completion of the acute i.v. morphine regime (i.e. neurones from the series of experiments described here in Chapter 1) were included in this particular comparison, hence the small group size (n=4).

The second point illustrated by this histogram is that for those neurones in the i.c.v. morphine-infused group which underwent a regime of i.v. morphine and went on to be tested with naloxone, firing rate was significantly elevated compared to the pre-naloxone firing rate (from 2.6Hz to 7.1Hz; P=0.02, 1-tailed paired t-test, n=4).

Figure 1.4.2.1.b) Percentage of control firing rate for both i.c.v. vehicle- and i.c.v. morphine-infused groups before and after naloxone

Data contributing to this histogram were derived solely from the experiments described here in Chapter 1.

In the i.c.v. vehicle-infused group there was no difference between the pre- and post-naloxone firing rate of the 5 neurones which were tested with naloxone (post-naloxone firing rate was 93.0% of pre-naloxone; n.s., unpaired 1-tailed t-test). However, the post-naloxone firing rate of the 4 neurones tested in the i.c.v. morphine-infused group was significantly greater than the post-naloxone firing rate of the 5 neurones measured in the i.c.v. vehicle-infused group (260.4% vs. 93.0% of their pre-naloxone control values respectively; P=0.0004, unpaired 1-tailed t-test).

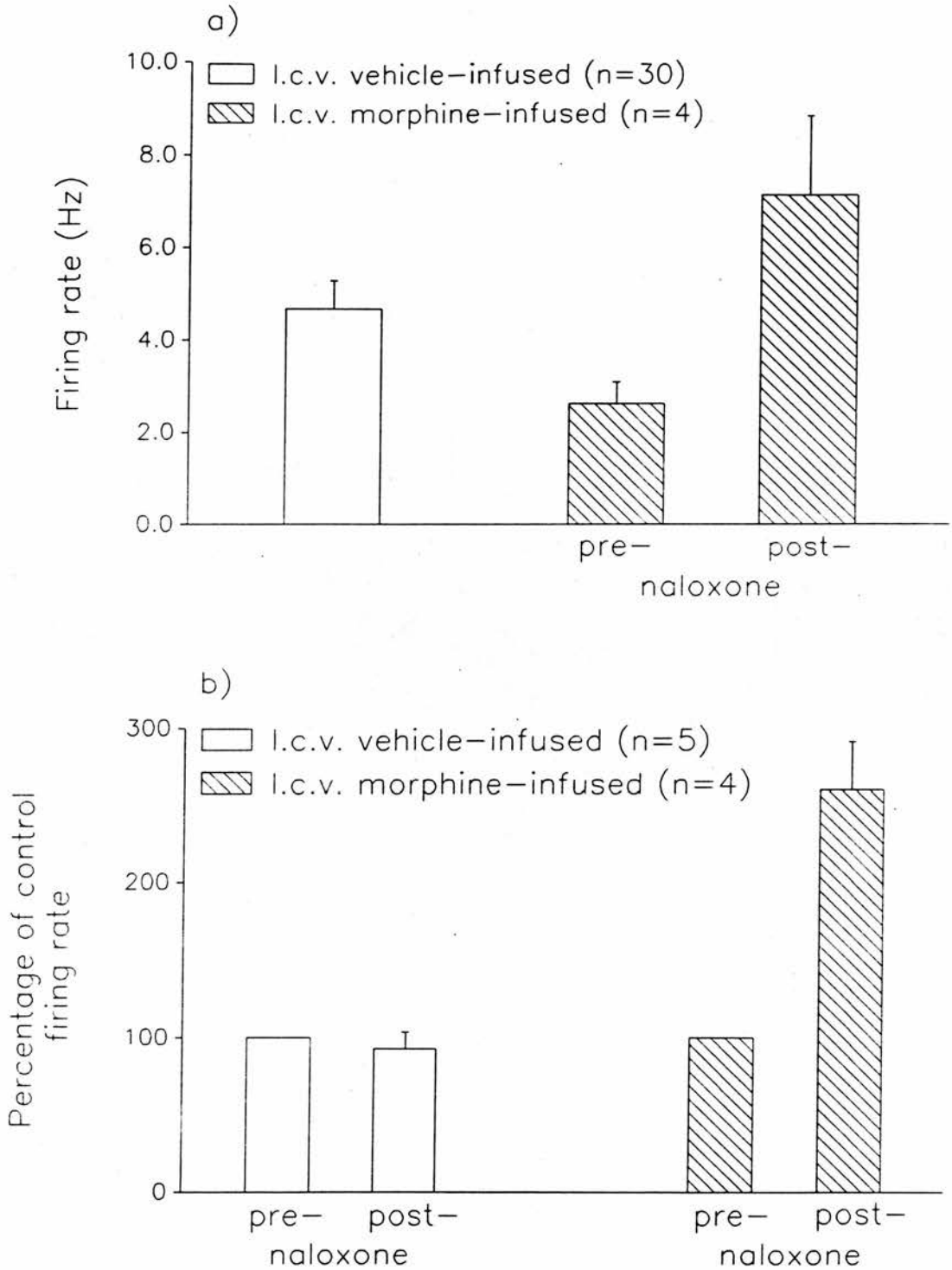
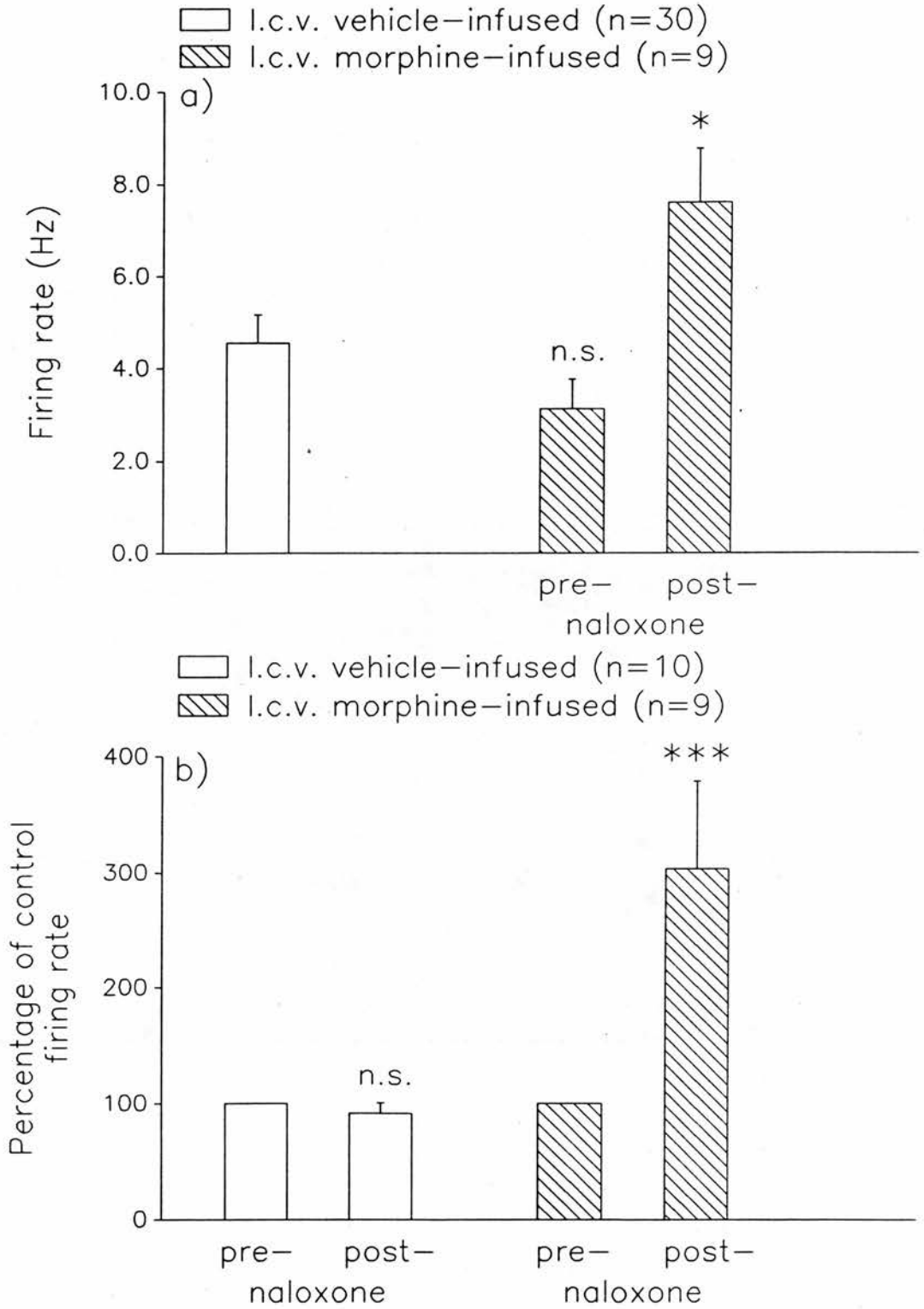

a)
10.0
8.0
6.0
4.0
2.0
0.0
Firing rate (Hz)
I.c.v. vehicle–infused (n=30)
I.c.v. morphine–infused (n=4)
pre–
post–
naloxone
b)
300
200
100
0
Percentage of control firing rate
I.c.v. vehicle–infused (n=5)
I.c.v. morphine–infused (n=4)
pre–
post–
naloxone
pre–
post–
naloxone

Figure 1.4.2.2. Comparison of pre- and post-naloxone firing rates

The comparison of pre- and post-naloxone firing rates in this figure includes cells drawn from a series of experiments using intravenous U50,488H (see Chapter 2) in place of morphine within the same protocol. Therefore in 5 of these cells naloxone is given after a series of doses of U50,488H.

a) Firing rate (Hz) of putative oxytocin neurones from 5-day i.c.v. vehicle-infused and 5-day i.c.v. morphine-infused rats. Firing rate is not different between vehicle- and morphine-infused groups (Wilcoxon). Pre- and post-naloxone firing rates are significantly different in the i.c.v. morphine-infused group (Wilcoxon, P=0.008). Post-naloxone firing rate is also different from the control firing rate for the i.c.v. vehicle-infused group (Wilcoxon, P=0.027).

b) Percentage of control firing rate for both i.c.v. vehicle- and i.c.v. morphine-infused groups before and after naloxone. Post naloxone firing rate is not different from control for the i.c.v. vehicle-infused group. As stated above, pre- and post-naloxone firing rates are significantly different from the i.c.v. morphine-infused group (Wilcoxon, P=0.008) and post-naloxone firing rate for the i.c.v. morphine-infused group is significantly greater than the post-naloxone firing rate in the i.c.v. vehicle-infused group (Wilcoxon, P=0.0007).


I.c.v. vehicle–infused (n=30)
I.c.v. morphine–infused (n=9)
a)
Firing rate (Hz)
10.0
8.0
6.0
4.0
2.0
0.0
*
n.s.
pre–
post–
naloxone
I.c.v. vehicle–infused (n=10)
I.c.v. morphine–infused (n=9)
b)
Percentage of control firing rate
400
300
200
100
0
***
n.s.
pre–
post–
naloxone
pre–
post–
naloxone

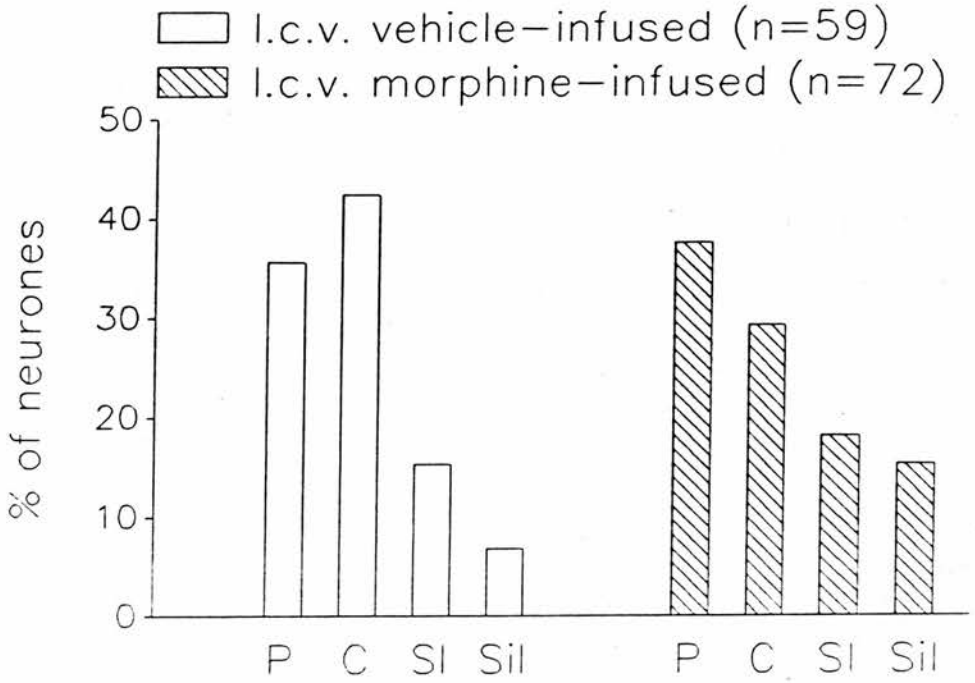


Figure 1.4.4.1. Comparison of the distribution of electrical activity in supraoptic nuclei from 5-day i.c.v. vehicle-infused rats and 5-day i.c.v. morphine-infused rats

The proportion of continuous and phasic neurones are similar in the i.c.v. vehicle-infused group. After chronic morphine infusion the proportion of neurones in all 4 categories was unchanged compared to the i.c.v. vehicle-infused group.

Abbreviations:-

P: phasic neurones

C: continuous neurones

SI: slow-irregular neurones

Sil: silent neurones

(After Poulain and Wakerley, 1982)

CHAPTER 2

THE EFFECTS OF U50,488H ON OXYTOCIN NEUROSECRETORY NEURONES IN MORPHINE-NAIVE AND CHRONIC I.C.V. MORPHINE-INFUSED RATS: DOES CROSS-TOLERANCE DEVELOP IN TERMS OF NEURONAL ACTIVITY?

2.1. INTRODUCTION

Subtypes of opioid receptors were first postulated by Martin in 1967 to explain the dual action of nalorphine in man, where it antagonised morphine analgesia but was analgesic itself. The existence of the κ-opioid receptor was thought to account for this observation (Martin, 1967). Opioid receptors fall into 3 subtypes, μ, δ and κ historically defined by different pharmacological profiles of opioid ligands in neurophysiological and behavioural tests in the spinal dog (Gilbert and Martin, 1976; Lord et al,1977) and their prototype ligands are morphine, DADLE (D-Ala^2, D-Leu^5-enkephalin) and ketazocine respectively. Each subtype of receptor binds endogenous opioids with varying degrees of affinity: β-endorphin, Met-enkephalin and Leu-enkephalin to the μ-receptor and dynorphin to the κ-receptor (Corbett et al, 1982; Kosterlitz and Paterson, 1985).

There are κ-opioid receptors in the SON (Clark et al, 1986; Mansour et al, 1986; Mansour et al, 1987; Tempel and Zukin, 1987) and we have found both μ- and κ-opioid receptors in the SON (Sumner et al, 1990; see Chapter 5). Both morphine (Pittman et al, 1980; Wakerley et al, 1983) and the κ-opioid receptor agonist, U50,488H (*trans*-(±)-3,4-dichloro-*N*-methyl-*N*-(2-[1-pyrrolidinyl]cyclohexyl) benzeneacetamide, methane sulphonate salt) acutely affect the electrical activity of oxytocin (OT)-secreting neurones (Russell et al, 1989b) and the OT-secreting system develops tolerance to chronic i.c.v. morphine (Bicknell et al, 1988a; Rayner et al, 1988; Sumner et al, 1989; Leng et al, 1990; see also Chapter 1).

Tolerance i.e. loss of effect of the opioid with repeated administration requiring higher doses to be used to produce the same initial effect, develops to selective ligands binding at all 3 receptor subtypes including to endogenous opioid peptides (Christie et al, 1982; Redmond and Krystal 1984; Sivam and Ho, 1984).

Cross-tolerance can occur between drugs acting at the same or at similar receptor sites and this is seen when a system made tolerant to one drug by chronic

administration exhibits tolerance to another drug to which the system has not previously been exposed. Cross tolerance involving two drugs is indicative of either a shared receptor site or of shared membrane/post-receptor mechanisms i.e. of some interaction between two distinct receptor sites (Wuster et al, 1983).

Morphine binds most avidly to the μ-opioid receptor and U50,488H to the κ-opioid receptor and both are high affinity agonists at their respective receptor sites with very little affinity for the other opioid receptor sites (Kosterlitz, 1985; Lahti et al, 1985; Clarke and Pasternak, 1988). Lack of cross-tolerance has been reported between U50,488H and morphine but does occur between morphine and less highly selective κ-opioid agonists such as ethylketocyclazocine and the prototype κ-opioid agonist ketazocine in mice (Von Voightlander and Lewis, 1982).

Tolerance has been described to the μ-opioid receptor agonist morphine when chronically administered in the rat by i.c.v. infusion, in terms of plasma OT concentration and in terms of the electrical activity of the OT-secreting neurones of the SON (Leng et al, 1990 and Chapter 1).

The question of whether tolerance to i.c.v. morphine in the rat confers tolerance to the κ-opioid receptor agonist U50,488H in terms of electrical activity of OT-secreting neurones of the SON is addressed in this Chapter. A preliminary account of this study has been published (Leng et al, 1990).

2.2. METHODS

2.2.1. Animals

Virgin female Sprague-Dawley rats were used; housed at 21-23°C and allowed food (standard breeder diet) and tap water ad libitum; they were kept under a 13h/11h light/dark cycle.

2.2.2. Preparation for electrophysiology

2.2.2.1. Control group

Under ether anaesthesia, virgin female Sprague-Dawley rats (weight: 273.0 ± 6.0g, n=19) were implanted with an i.c.v. cannula into the right lateral cerebral ventricle, which was attached to an infusion assembly consisitng of a polythene cannula and osmotic minipump (Alzet 2001, Alza Corp.), filled with sterile pyrogen-free water (vehicle) to be infused at 1μl/h for 5 days. Full details are contained in Chapter 1., Section 1.2.1..

2.2.2.2. Chronic i.c.v. morphine-treated group

Virgin female Sprague-Dawley rats (weight: 268.0 ± 6.8g, n=17) were anaesthetised with ether and cannulated with an i.c.v. chronic infusion assembly which delivered morphine sulphate solution (in sterile pyrogen-free water) over 5 days in increasing dose (10μg/h for 40h, 20μg/h for 40h and 50μg/h for 40h). See Chapter 1, Section 1.2.2. for full details.

2.2.3. Electrophysiology

Methodology was as detailed in Chapter 1 in most respects. Briefly, On the sixth day of i.c.v. morphine infusion, rats were anaesthetised with urethane (ethyl carbamate, 1.25g/kg i.p.) and the trachea and a jugular vein cannulated. The right SON and pituitary stalk were exposed by ventral surgery and a concentric bipolar stimulating electrode placed on the pituitary stalk to antidromically-activate SON neurones projecting to the the posterior pituitary (ventral surgery performed by Dr G. Leng). Extracellular recording of spontaneous and antidromically-activated electrical

activity was made with 0.9% NaCl-filled glass micropipettes (20-40MOhm). Putatively oxytocinergic neurones were selected for experiment and tested with i.v. U50,488H (in 0.9% NaCl, diluted to give an injection volume of 500μl/kg; a gift from Upjohn or from Sigma Chemical Co.) given in increasing dose (1, 10, 50, 100, 500, 1000μg/kg up to a maximum dose of 5mg.kg) at 10 min intervals and when appropriate, naloxone hydrochloride (10mg/ml, 5mg/kg) was injected to end the protocol. Determination of threshold effective dose was made and the results treated in the same way a those given in Chapter 1.

2.2.4. Blood sampling

Virgin female Sprague Dawley rats (weight: control group (received no low doses of U50,488H), 269.4 ± 4.1g, n=10; experimental group (received low and high doses of U50,488H), 262.5 7.6g, n=10) were anaesthetised with urethane (1.25g/kg i.p.) and a femoral vein and artery cannulated for injection of U50,488H or saline and for blood sampling (0.25ml into a heparinised syringe). After 2-3 hours equilibration, the protocol was started. The protocol is illustrated in Table 2.2.7.. Samples 2, 4 and 6 were followed immediately with a dose of U50,488H in 500μl/kg or with the same volume of 0.9% saline according to the schedule. The doses of U50,488H were selected to test possible stimulatory effects of low doses. Rats were assigned at random to the control or experimental groups (n=10 for each group). Samples were collected into 1.5ml Eppendorf tubes were centrifuged to separate off plasma which was transferred into 1.5ml Eppendorf tubes and frozen at -40°C for later radioimmunoassay of oxytocin content.

2.2.5. Radioimmunoassay

The full method used for oxytocin radioimmunoassay is contained in Chapter 4, section 4.2.2.1. and calculation of results in 4.2.2.3.. The method used was a modification of that described by Higuchi (Higuchi et al, 1985) and was expertly carried out by Chris Chapman, Dept. Neuroendocrinology, AFRC, Institute of Animal

Physiology and Genetics Research, Babraham, Cambridge. The sensitivity of the assay was 0.2-0.4pg/tube. The intra-assay coefficient of variation (the sample standard deviation expressed as a percentage of the sample mean) was calculated from:

$$\sigma/\mu \times 100,$$

where σ=sample standard deviation and μ=sample mean of all intra-assay standards. In this case the intra-assay coefficient of variation was 7.6%.

2.2.6. Statistics

Statistical comparison of groups was performed as for vehicle-infused and morphine-infused groups in Chapter 1, by the Wilcoxon test for independent samples and by comparison of the proportion of neurones inhibited at a given dose.

Comparison of pre- and post-naloxone firing rate was made with Student's paired t-test, one- and two-tailed. Where firing rate was expected to rise after naloxone, according to previously published reports, I thought it justified to use a one-tailed comparison. Where no hypothesis existed I applied a two-tailed t-test. The results of both one- and two-tailed comparisons are given in the Results section even where a one-tailed comparison was the test of choice. Student's unpaired two-tailed or one-tailed t-test was used to compare the percentage change in firing rate after naloxone between the i.c.v. vehicle-infused and the i.c.v. morphine-infused groups.

Because of small sample sizes for the comparison of pre-/post-naloxone effects, data were pooled with those from Chapter 1, (where rats received acute i.v. morphine instead of i.v. U50,488H) and any changes tested. The results for both the original and the pooled data were expressed (see Results section).

Where Student's t-test was used to make comparisons the samples were all tested for normality by comparison with a theoretical normal distribution with the Kolmogorov-Smirnov one sample test. All samples were normally distributed according to this test.

Table 2.2.7. Blood sampling protocol: the effects of i.v. U50, 488H on oxytocin secretion

Time (mins)	Procedure	Injectate (500μl/kg)	
		control	U50,488H
0	Sample 1		
10	Sample 2 *Dose 1*	0.9% saline	1μg/kg U50,488H
14	Sample 3		
20	Sample 4 *Dose 2*	0.9% saline	10μg/kg U50,488H
24	Sample 5		
30	*Dose 3*	1mg/kg U50,488H	1mg/kg U50,488H
40	Sample 6 *Dose 4*	0.9% saline	5mg/kg naloxone
50	Sample 7		

2.3. RESULTS

2.3.1. Characteristics of supraoptic non-phasic putative oxytocin neurosecretory neurones

2.3.1.1. Vehicle-infused rats

This summary of observations on general activity was obtained from data pooled between this study and that in Chapter 1 and the proportion of cell types recorded, mean firing rate, latency to antidromic activation and threshold voltage to antidromic activation are all detailed in Chapter 1, Section 1.3.1.1..

2.3.1.2. Morphine-infused rats

The summary of these data is detailed in Chapter 1, Section 3.1.2..

2.3.2. Characteristics of supraoptic phasic putative vasopressin neurosecretory neurones

The summary of these data is given in Chapter 1, Section 3.2..

2.3.3. Effects of i.v. U50,488H on supraoptic non-phasic putative oxytocin neurones in i.c.v. vehicle-infused rats (see Figure 2.3.3.1.)

Both excitatory and inhibitory effects of i.v. U50,488H were seen according to dose. The cumulative threshold dose of U50,488H to inhibit significantly 7/8 putative oxytocin neurones that responded was from 1μg/kg to 1661μg/kg (median dose: 11μg/kg, 8 cells). The mean ± SE firing rate of these 7 neurones was 4.85 ± 1.4 Hz. The control firing rate of the neurone that did not respond was 1.3 Hz (Figure 1.h.) and thus was not identified as putatively oxytocinergic or vasopressinergic. The mean ± SE firing rate of all 8 neurones in this group was 4.4 ± 1.3 Hz. The effect of U50,488H was evident within 30 seconds of injection.

The highest doses used varied according to the responsiveness of individual cells to U50,488H and was between 161μg/kg and 6661μg/kg. Where the cumulative dose exceeded 661μg/kg (i.e. the cell was kept for most of the protocol) firing rate was reduced by a mean ± SE of 94.0 ± 2.4% (n=5 cells; 2 other cells were maximally

inhibited at less than 661μg/kg; 1 cell already described above, was not inhibited even at the maximal dose used).

At low doses of U50,488H excitatory effects were seen in 3/8 cells tested for cumulative threshold dose to excitation with U50,488H at 1-10μg/kg. The remaining cells showed either no change from the control firing rate over this dose range, or inhibition. Of 3 further cells which did not contribute to the estimation of threshold cumulative dose to inhibition because the recordings were lost early on in the protocol, 2 were excited by U50,488H at a threshold dose of 1μg/kg whilst one cell was neither excited nor inhibited at 1μg/kg. Thus, in total, 5/11 cells tested were excited by low doses of U50,488H. Cells excited by U50,488H in low doses were always inhibited at higher doses where tested, indicating a biphasic response.

Whilst 3/8 cells in this group (5/11 cells overall) were excited by U50,488H at 1-10μg/kg (3 at 1μg/kg and 2 at 10 μg/kg overall), 5/8 cells (5/11 cells overall) were significantly inhibited in this dose range (2 at 1μg/kg and 3 at 10μg/kg).

In those cells tested with naloxone HCl (5mg/kg) after inhibitory doses of U50,488H had been given, firing rate was restored to 90.3 ± 14.8% (n=5) of the control firing rate. There was no evidence of overshoot of the firing rate to beyond that seen in the control period.

Figure 2.3.3.1.(overleaf) I.c.v. vehicle-infused group: summary of firing rate changes

Each histogram is the record of one neurone in one rat which had received an i.c.v. infusion of vehicle over the previous 5 days and was then urethane-anaesthetised for extracellular recording from identified neurones in the SON.

Firing rate of putative OT neurones is expressed as a percentage of the control firing rate measured in 30sec bins during a control period of at least 10minutes. The intravenous dose of U50,488H is given on the horizontal axis in μg/kg and ranges from 1μg/kg to 5000μg/kg at 10minute intervals. Inhibition was defined as the firing rate during any 10minute drug treatment period for which the 95% confidence bands about the mean did not overlap with those of the control period. Statistically significant inhibitions are marked with an asterisk. The control period firing rates were a) 4.4Hz b) 3.0Hz c) 11.75Hz d) 0.9Hz e) 1.8Hz f) 7.2Hz g) 4.9Hz h) 1.3Hz. All cells except one, (h), were significantly inhibited by 161μg/kg U50,488H or less. At low doses U50,488H was excitatory (see cells f), g) and h)).and this effect was reversed in two of these cells tested at higher doses (f) and g)).

Naloxone (NAL) where given fully reversed the inhibitory effects of U50,488H but did not result in overshoot in activity.

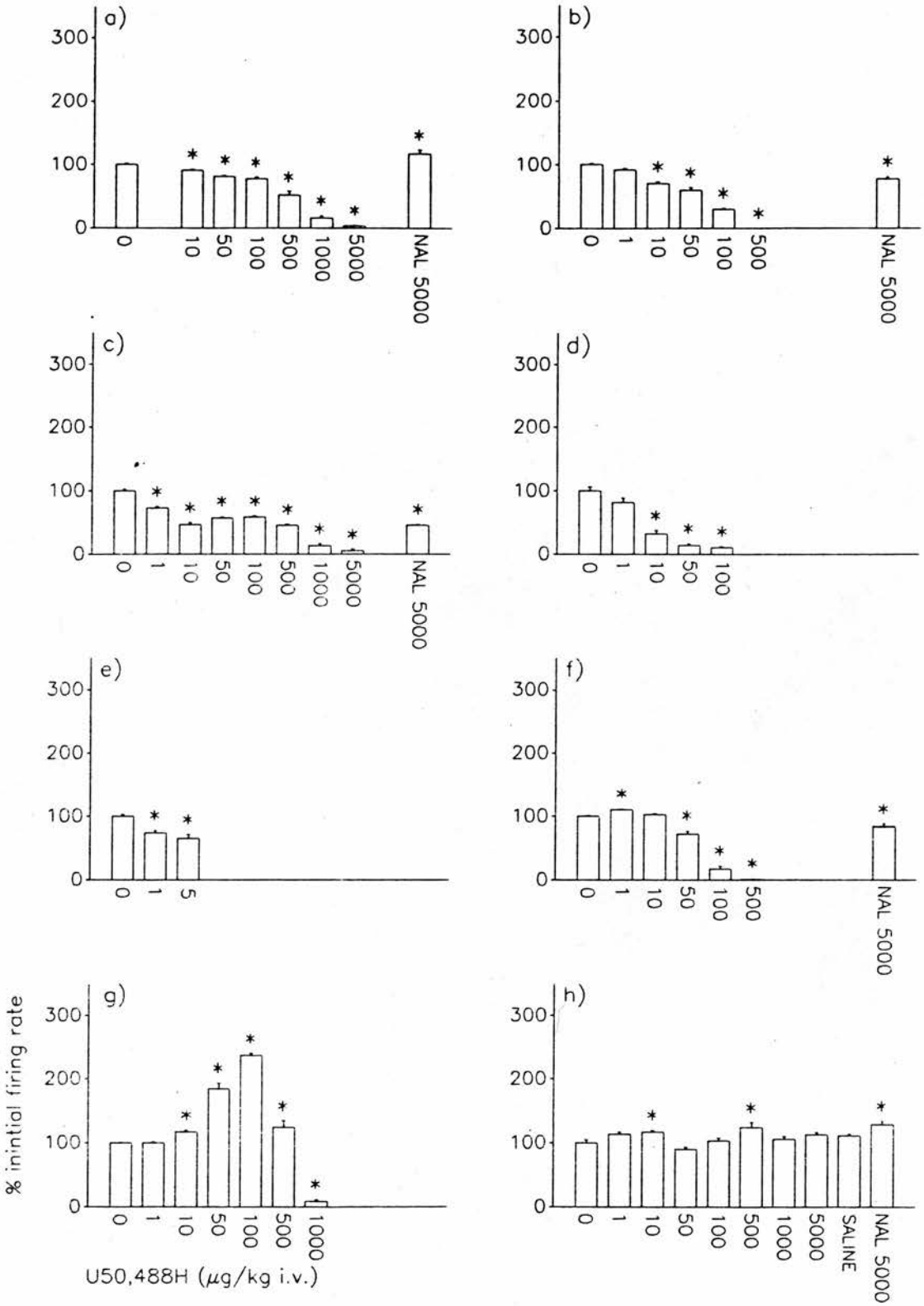

a)
b)
c)
d)
e)
f)
g)
h)
% inintial firing rate
U50,488H (μg/kg i.v.)
0
1
5
10
50
100
500
1000
5000
NAL 5000
SALINE
300
200
100

Figure 2.3.3.1.

2.3.4. Effects of U50,488H on plasma oxytocin concentration in normal rats

U50,488H was not effective in decreasing the plasma oxytocin concentration within the dose range investigated (see Figure 2.3.4.1.).

U50,488H produced no measurable effect on basal oxytocin secretion under urethane anaesthesia at 1μg/kg i.v. compared to the first 2 control samples in this group or compared to the equivalent plasma sample in the control group (Wilcoxon test for independent samples).

Injection of U50,488H at 10μg/kg resulted in a rise in plasma oxytocin concentration to 42.10 ± 5.66pg/ml, 475.8% pre-U50,488H control (8.85pg/ml) concentration (P=0.004, significantly different from its pre-U50,488H control value, Wilcoxon signed rank test). The next dose of U50,488H, 1mg/kg, given to both groups resulted in no change in the control group against its own control value but in the group already given the low doses of U50,488H, plasma oxytocin was still increased compared to the pre-U50,488H control value (P=0.027, Wilcoxon signed rank test). However, after 1mg/kg U50,488H plasma oxytocin concentration did not differ significantly between groups.

Finally, naloxone (5mg/kg) given to the group given low and high doses of U50,488H alone, resulted in a rise in plasma oxytocin which was significantly greater than its pre-U50,488H control value (P=0.004, Wilcoxon signed rank test) and from the equivalent sample from the control group (P=0.0008, Wilcoxon test for independent samples) given isotonic saline instead.

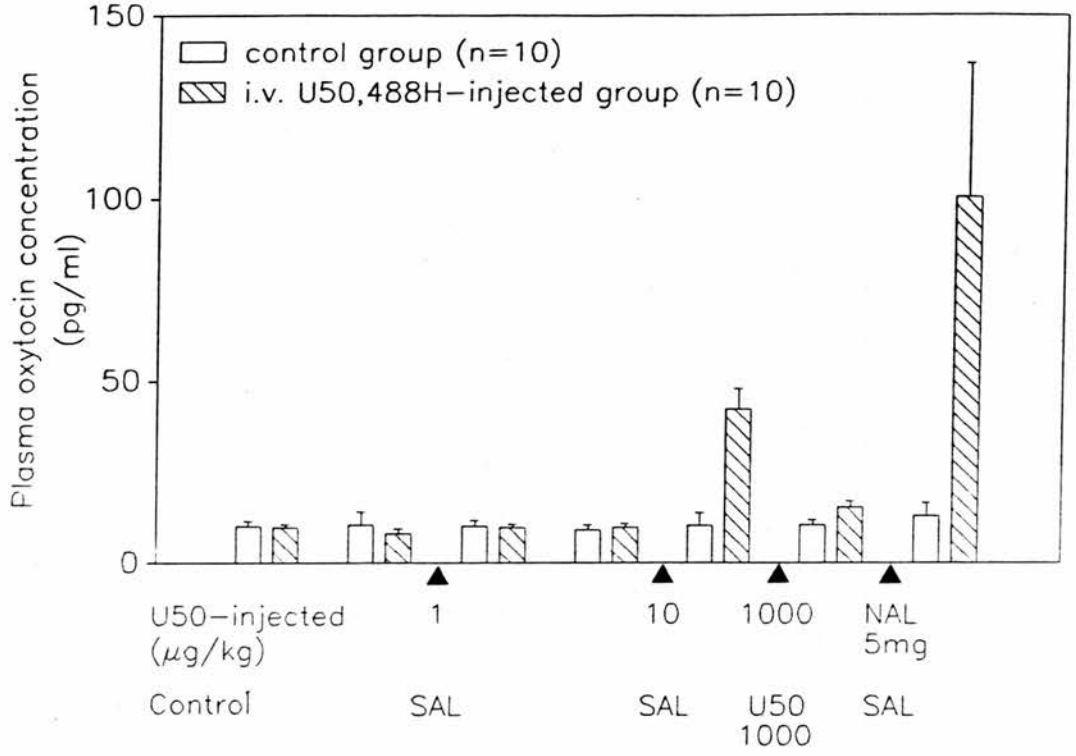


Figure 2.3.4.1. Effects of U50,488H on plasma oxytocin concentration in normal rats

Virgin female Sprague Dawley rats were anaesthetised with urethane and blood sampled via a femoral artery (0.25ml sample volume). Injections were made via a femoral vein and injection volume was 500μl/kg. The y-axis denotes plasma oxytocin concentration (pg/ml) as measured by radioimmunoassay. The 'control' group was injected with only one (high) dose of U50,488H (diluted in 0.9% saline) and 0.9% saline in place of the lower doses of U50,488H **and in place of naloxone,** according to the protocol in the Methods Section of this Chapter. The U50,488H-treated group was injected with U50,488H in increasing dose and finally with naloxone (5mg/kg). Plasma oxytocin increased significantly in the U50,488H-treated group after 10μg/kg U50,488H. Naloxone provoked a statistically-significant rise in plasma oxytocin concentration in the U50,488H-treated group (P=0.004 vs. its pre-U50,488H control value, Wilcoxon signed rank test) but not in the control group.

Abbreviations: SAL: 0.9% saline; NAL: naloxone; U50: U50,488H.

2.3.5. Effects of i.v. U50,488H on supraoptic non-phasic, putative oxytocin neurones in i.c.v. morphine-treated rats (see Figure 2.3.5.1.)

The mean ± SE basal firing rate of these neurones was 3.0 ± 0.9 Hz (n=8; not significantly different from the i.c.v. vehicle-treated group, Wilcoxon). Of this group, 5 neurones fired continuously (mean ± SE firing rate 4.4 ± 0.9 Hz) and 3 were slow-irregular cells (mean ± SE firing rate 0.8 ± 0.06 Hz).

U50,488H inhibited 7/8 of these non-phasic neurones. These 7 cells were inhibited over a range of threshold cumulative dose of i.v. U50,488H of 11μg/kg to 1661μg/kg. The median threshold cumulative dose of i.v. U50,488H to inhibition was between 161μg/kg and 661μg/kg U50,488H) in 8 cells (not significantly different from the i.c.v. vehicle-treated group, Wilcoxon test for independent samples).

At the highest dose of i.v. U50,488H used, 6.661mg/kg, the firing rate was reduced by 70.3 ± 18.1% (mean ± SE; n=5 cells, not significantly different from the i.c.v. vehicle-infused group, Wilcoxon test for indpendent samples).

At low doses of i.v. U50,488H excitatory effects were seen in 4/8 non-phasic cells (to 181.1 ± 29.7% control mean firing rate at a threshold dose of 1, 1, 11, 61μg/kg). Of these cells however only one (Figure 2.3.5.1.e)) remained excited at the threshold cumulative dose to inhibition for the morphine-infused group and this cell was excited more so than any other cell in this group, to 267.4% control firing rate at the threshold cumulative dose to excitation, 1μg/kg. It was inhibited significantly at 1661μg/kg U50,488H and was profoundly inhibited by 6661μg/kg by 99.4%. The dose response relation shown in Figure 2.3.5.2. suggests that there is a difference in responsiveness between the two groups, but looking closely at the threshold response of neurones reveals the explanation. Where cells were excited by U50,488H at low threshold doses (1-10μg/kg for the vehicle-infused group and 1-61μg/kg for the morphine-infused group) this was not reversed to inhibition until 50 and 1000μg/kg (2 cells) for the vehicle-infused group and 500, 1000 and 1000μg/kg (3 cells) for the morphine-infused group. This contributes to the slight shift in the threshold response to

inhibition for the morphine-infused group which nevertheless is not statistically significant.

Figure 2.3.5.3. illustrates the difference in response between the acute i.v. morphine-treated (see Chapter 1) and the acute i.v. U50,488H-treated chronic i.c.v. morphine-infused groups at 161μg/kg. Where 8/8 neurones failed to respond to acute morphine at this dose, the majority of neurones tested were inhibited significantly by U50,488H indicating no effect of chronic i.c.v. morphine on the responsiveness of non-phasic neurones to the κ-receptor agonist, U50,488H while a profound effect was seen on the responsiveness to acute morphine.

Naloxone (5mg/kg i.v.) produced withdrawal excitation compared with the pre-U50,488H control mean firing rate. In the first 10 minutes after naloxone mean firing rate was increased to 337.4 ± 138.8% of mean control firing rate ($P < 0.03$, Student's paired one-tailed t-test, n=5) whereas the firing rate after naloxone in the i.c.v. vehicle-infused group was 90.3% control firing rate (not significantly different, Student's paired two-tailed t-test). The percentage change in firing rate after naloxone was not significantly different between the two groups however (P=0.057, Student's unpaired one-tailed t-test). This comparison suffers from small sample sizes as not all cells could be tested with naloxone.

When data from the rats which received acute i.v. morphine (Chapter 1) instead of i.v. U50,488H were pooled with the present data, and the comparison of the pre-/post-naloxone change in firing rate made between these enlarged groups, they were shown to be different (P=0.009, Student's unpaired two-tailed t-test; P=0.004, Student's unpaired one-tailed t-test). Likewise, the degree of statistical significance achieved for the comparison of pre-/post-naloxone firing rate in the i.c.v.-morphine-infused group was greater (P=0.002, Student's paired two-tailed t-test; P=0.001, Student's paired one-tailed t-test) when data were pooled but the comparison of pre-/post-naloxone firing rate in the i.c.v. vehicle-infused group still did not attain significance (P=0.12, Student's paired two-tailed t-test).

Figure 2.3.3.1.(overleaf) I.c.v. vehicle-infused group: summary of firing rate changes

Each histogram is the record of one neurone in one rat which had received an i.c.v. infusion of vehicle over the previous 5 days and was then urethane-anaesthetised for extracellular recording from identified neurones in the SON.

Firing rate of putative OT neurones is expressed as a percentage of the control firing rate measured in 30sec bins during a control period of at least 10minutes. The intravenous dose of U50,488H is given on the horizontal axis in μg/kg and ranges from 1μg/kg to 5000μg/kg at 10minute intervals. Inhibition was defined as the firing rate during any 10minute drug treatment period for which the 95% confidence bands about the mean did not overlap with those of the control period. Statistically significant inhibitions are marked with an asterisk. The control period firing rates were a) 4.4Hz b) 3.0Hz c) 11.75Hz d) 0.9Hz e) 1.8Hz f) 7.2Hz g) 4.9Hz h) 1.3Hz. All cells except one, (h), were significantly inhibited by 161μg/kg U50,488H or less. At low doses U50,488H was excitatory (see cells f), g) and h)).and this effect was reversed in two of these cells tested at higher doses (f) and g)).

Naloxone (NAL) where given fully reversed the inhibitory effects of U50,488H but did not result in overshoot in activity.

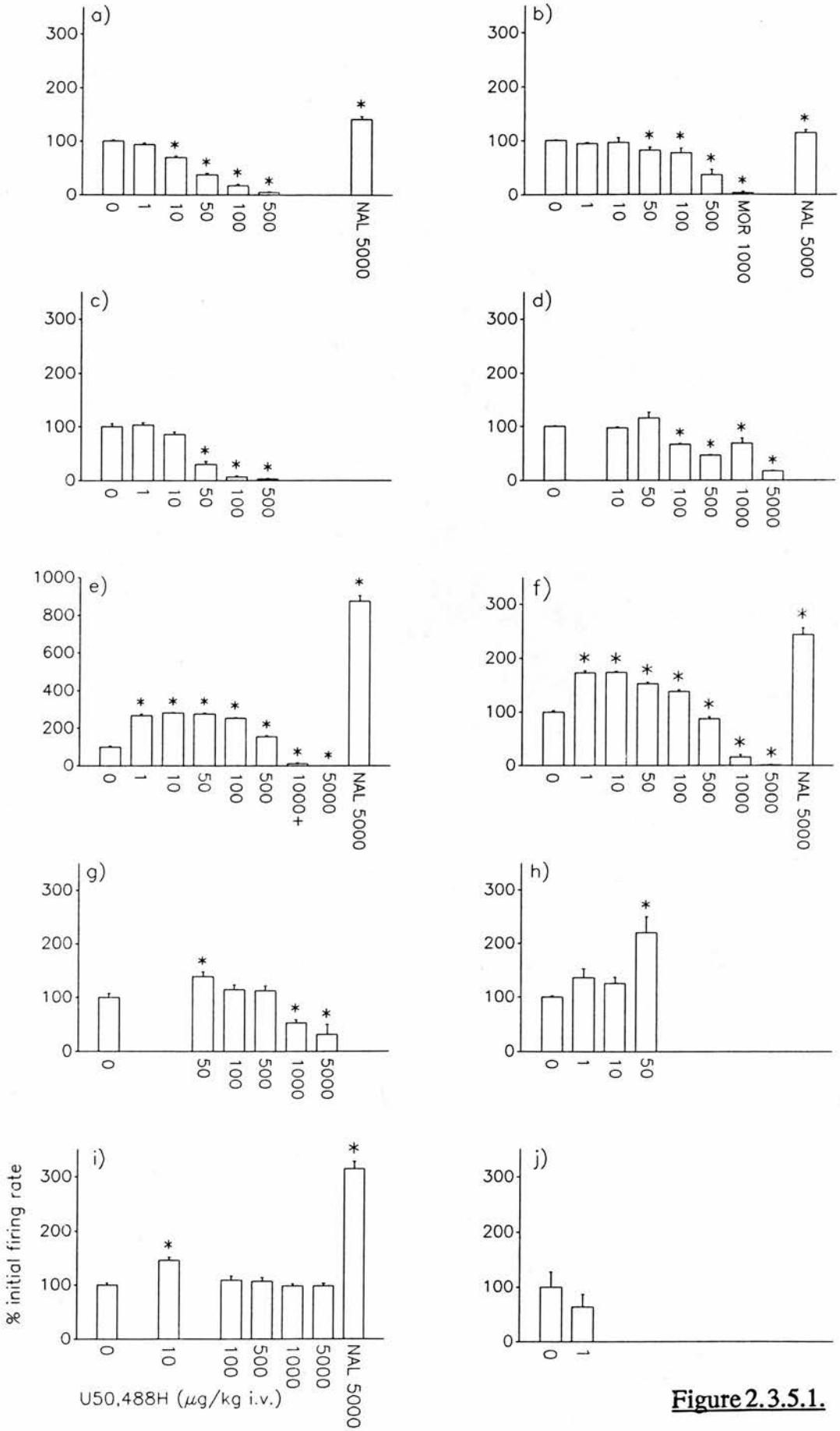

a)
b)
c)
d)
e)
f)
g)
h)
i)
j)
% initial firing rate
U50,488H (μg/kg i.v.)
NAL 5000
MOR 1000

Figure 2.3.5.1.

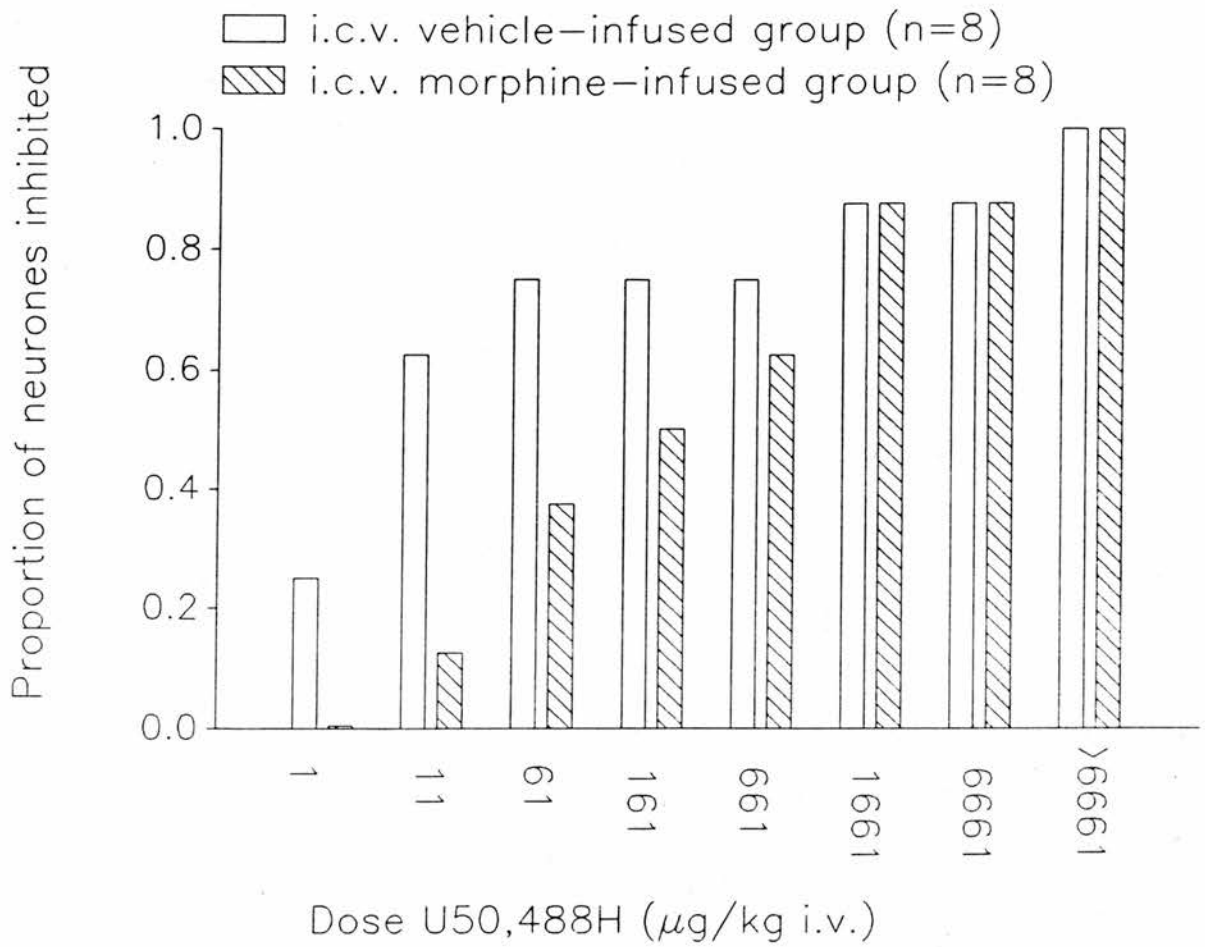


Figure 2.3.5.2. Comparative dose response relations for supraoptic putative oxytocin neurones from 5-day i.c.v. vehicle-infused and 5-day morphine-infused rats

Horizontal axis denotes cumulative dose of U50,488H (μg/kg i.v.) and the vertical axis, the proportion of neurones in each group which were inhibited by a given i.v. dose of U50,488H. The responsiveness of neurones recorded in chronic i.c.v. vehicle-infused rats to i.v. U50,488H was not different from that in rats chronically i.c.v. infused with morphine as indicated by their median threshold inhibitory doses which were not different (Wilcoxon). The apparent lack of response in the chronic i.c.v. morphine-infused group to low doses of U50,488H is explained by the occurrence of low-dose excitation such that relatively high doses of U50,488H were required to subsequently inhibit electrical activity (see text).

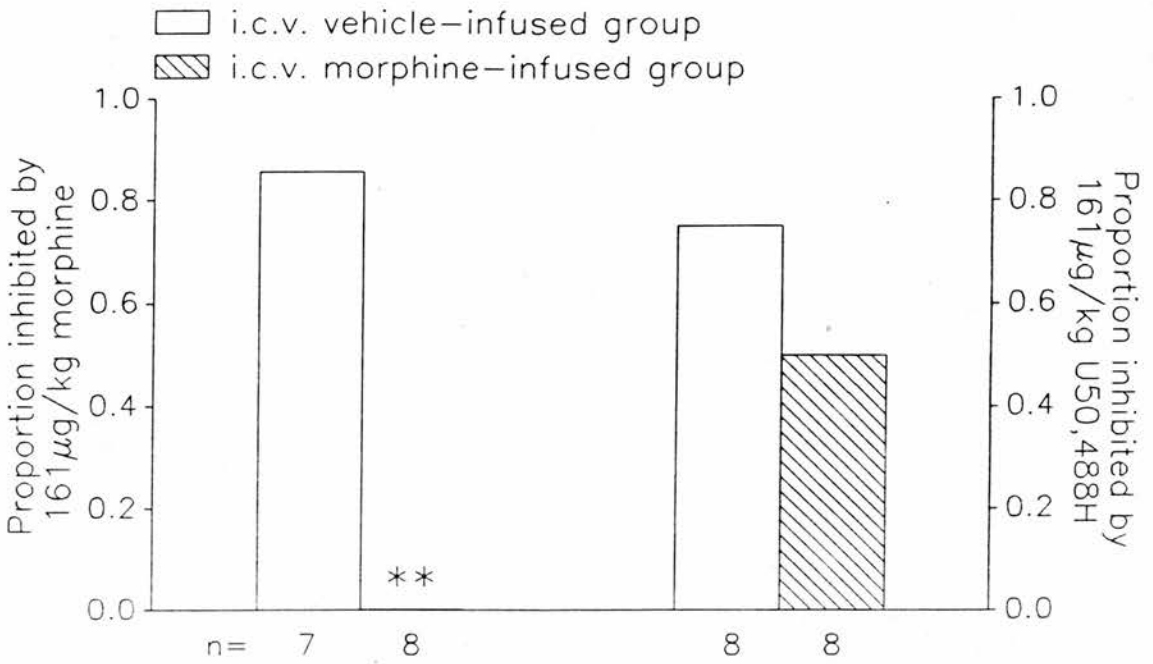


Figure 2.3.5.3. Comparison of the proportion of putative oxytocin neurones inhibited by 161μg/kg of either i.v. morphine of i.v. U50,488H in chronic i.c.v vehicle-infused and chronic i.c.v. morphine-infused rats

These data are derived from both Chapters 1 and 2. A comparison is made of the effectiveness of i.v. morphine (a μ-opioid receptor-selective agonist) and i.v. U50,488H (a κ-opioid receptor-selective agonist) both at 161μg/kg to inhibit the electrical activity of putative OT neurones in morphine-naive and chronic i.c.v. morphine-infused rats. Whilst i.v. morphine loses its inhibitory potency at this dose in rats treated chronically with morphine (due to tolerance) U50,488H remains as effective in both morphine-naive and chronic i.c.v. morphine-infused rats, indicating that cross-tolerance has not developed to the κ-selective agonist U50,488H (see text). n denotes group size.

2.4. DISCUSSION

2.4.1. Technical considerations

Details of difficulties associated with the recording procedure are given in the Discussion section, Chapter 1. The procedure which enables neurones recorded part way through an interrupted protocol to be used in assessment of threshold inhibitory/excitatory dose of U50,488H is as described in the Discussion section of Chapter 1. In the vehicle-infused group, three cells fell into this category: a), g) and h). The control period of cell a) was recorded at least 50 minutes after the injection of U50,488H at 1μg/kg. Cell g) was recorded more than 40 minutes after the injection of U50,488H at 1μg/kg. Cell h) was recorded after 11μg/kg U50,488H 47 minutes previously. All three cells were considered to be either unaffected or sufficiently recovered from any effect of such low doses of U50,488H as to be within the limits of the control firing rate.

2.4.2. Effects of i.v. U50,488H on supraoptic putative oxytocin neurones from i.c.v. vehicle-infused rats

Acute i.v. U50,488H given to i.c.v. vehicle-infused rats was sometimes excitatory at low doses and was inhibitory at higher doses. The threshold cumulative inhibitory dose of U50,488H was 11μg/kg i.v. and this compares well with that for i.v. morphine in vehicle-infused rats, <= 60μg/kg in those cells affected, as described in the previous Chapter. There are κ-type opiate binding sites in the SON defined by autoradiography (Mansour et al, 1986; Tempel and Zukin, 1987; Mansour et al, 1988; Sumner et al, 1990) as well as dynorphin binding sites (Clark et al, 1986). U50,488H is a highly selective ligand at the κ-opioid receptor with a poor affinity for μ- and δ-opioid receptors (Von Voightlander et al, 1983; Kosterlitz, 1985; Clarke and Pasternak, 1988; Leff and Dougall, 1989) but U50,488H will activate μ-opioid receptors at high concentrations and under suitable conditions (Leff and Dougall,1989). The putative endogenous ligand for the κ-opioid receptor is dynorphin

(Corbett et al, 1982; Gerstberger and Barden,1986) which has been found in the SON by immunocytochemical (Watson et al, 1982) and in situ hybridisation (Lightman and Young, 1987) studies. Dynorphin has also been shown to be inhibitory to supraoptic neurones in vitro in the rat hypothalamic slice preparation and we have shown that U50,488H is inhibitory to these neurones, again in the hypothalamic slice (Russell et al, 1989b). So, it was not surprising to discover that U50,488H was inhibitory to these neurones in this preparation.

More surprising was the incidence and the degree of low dose excitation seen with U50,488H. In those cells excited by U50,488H at a low dose (1-10μg/kg, 3/8 cells), inhibition was always produced at a higher dose where tested, indicating a biphasic effect of the κ-receptor agonist on factors controlling the electrical activity of supraoptic non-phasic neurones. Opiates can be indirectly and, contrary to long held opinions, directly excitatory to neurones. Indirect excitation is achieved by opioid inhibition of an inhibitory synaptic drive as occurs in the hippocampus (Ziegelgansberger et al, 1979; Robinson and Deadwyler, 1981; Siggins and Ziegelgansberger, 1981; Bradley and Brookes, 1984; Neumaier et al, 1988; Swearengen and Chavkin, 1989). There is a well documented inhibitory GABAergic input to the neurones of the SON (Randle et al, 1986; Theodosis et al, 1986; Buijs et al, 1987; Renaud, 1987; Sakaue et al, 1988) which might be inhibited by U50,488H, but an interaction between opioids and GABA synapses has not been demonstrated for these neuroendocrine cells.

The second possible scenario, direct opioid excitation, arises from electrophysiological studies of dorsal root ganglion cells in culture where prolongation of action potential duration by low (nanomolar) concentrations of μ- and δ-opioid agonists occurs by a direct action, as synaptic connections are not present. The effects of μ- and δ- agonists are attributed to a decrease in a voltage sensitive K^+ conductance whereas the prolongation of the action potential by the κ-opioid agonist U50,488H is apparently the result of an increase in a voltage sensitive Ca^{2+} conductance. If the

change in duration of the action potential is interpreted in terms of its effect upon the voltage-sensitive calcium entry necessary for neurotransmitter release from nerve terminals, then the prolongation or shortening of the action potential can be thought of as evidence of "excitation" or "inhibition" respectively (see Chapter 1, p56 for more detail; for review see Crain and Shen, 1990). In these cells the "excitatory" and the "inhibitory" effects seem to be mediated by modulation in opposite directions of a given current. That is, the action potential prolongation by μ-opioids at nanomolar concentrations is due to the impairment of a K^+ current whilst at micromolar concentrations μ-opioids increase the same K^+ current. Similarly, κ-opioids at nanomolar doses facilitate a Ca^{2+} current whilst at micromolar doses inhibit that same current (Werz and Macdonald, 1984b; Werz and Macdonald, 1985; North, 1986; Shen and Crain, 1989; Shen and Crain, 1990b). The excitatory component of this biphasic response to opioids cannot be dissociated: excitation by an opioid is always followed at higher concentrations by inhibition in these neurones. Such mechanisms have not been studied in SON neurones.

Although no evidence is available concerning identification of possible opioid receptor subtypes which might mediate this biphasic response, the antagonism of the response by 1-10nM naloxone suggests that opioid receptors do mediate both the excitatory and the inhibitory components (Shen and Crain, 1989). Evidence for two types of opioid receptor or one receptor linked to two distnict post-receptor mechanisms, mediating the excitatory and the inhibitory responses comes from work once again in dorsal root ganglion cells in which opioid induced prolongation of the action potential is blocked by Cholera toxin which ADP-ribosylates the stimulatory GTP-binding protein (G-protein), Gs (Shen and Crain, 1990a; Sawchenko and Gerfen, 1985). Similarly, the shortening of the action potential by opioids at higher concentrations involves a G-protein, but this time Gi or Go (two different inhibitory G-proteins which are sensitive to pertussis toxin). This is shown by the prevention of opioid induced action potential shortening by pertussis toxin which ADP-ribosylates

Gi and Go. Adding to this picture, a cAMP-dependent protein kinase in dorsal root ganglion cells mediates DADLE-induced prolongation but not shortening of the action potential (Chen et al, 1988). The implication is that excitatory events produced by opioids in these sensory neurones are mediated by receptors positively coupled via a Gs-type G-protein to adenylate cyclase and cAMP-dependent voltage-sensitive ionic conductances leading to shorter duration action potentials and a propensity for increased firing rate, whereas inhibitory effects of opioids are mediated by opioid receptors linked to Gi or Go and voltage-sensitive ionic conductances which are probably not linked to changes in intracellular cAMP.

U50,488H does not alter the intracellular waveform of action potentials in locus coeruleus neurones in the range 0.01-1μM. The EPSP resulting from stimulation of afferent inputs was depressed and no IPSP was elicited by U50,488H suggesting a presynaptic site of action on excitatory afferent terminals. No biphasic response was seen (McFadzean et al, 1987).

Morphine (100pM-10nM) certainly affects the calcium-dependent potassium conductance in myenteric plexus neurones, where it is reported to prolong the after-hyperpolarisation following an evoked train of action potentials in a naloxone-reversible way (Tokimasa et al, 1981) but examination was not made of the effect of morphine on the shape and duration of the spontaneous action potential itself in this study.

Perhaps modulation of the action potential duration occurs in supraoptic neurones thus accounting for the excitatory effects seen there with low doses of U50,488H. This phenomenon was not investigated in depth in the present studies as recordings were extracellular and changes in action potential duration do occur with changes in firing rate in supraoptic neurones (Andrew and Dudek, 1985; Bourque and Renaud, 1985) making it difficult to differentiate the effect of U50,488H on action potential duration directly from any indirect effect following excitation or inhibition of firing. But there was no discernable difference in action potential duration between groups.

An earlier report, again using extracellular recording techniques in vivo reported no gross changes in the action potential duration after chronic morphine (Bicknell et al, 1988a). Comparison of extracellular action potential duration before and after application of U50,488H where this induced a significant excitation, revealed no discernible change in these neurones and so does not occur where excitation is clearly demonstrated.

There is no further evidence to support or refute the existence of spike-broadening at low doses and shortening at higher doses of opiates from intracellular recordings. Indeed, this finding in dorsal root ganglion is not supported by similar results in hippocampus with κ-opioid receptor agonists (Bradley and Brookes, 1984).

Additionally, there is as yet no evidence in the OT-secreting neurones of the SON for the presence of the subtypes of opiate receptor proposed to account for the biphasic effect. There are opioid κ-type receptors in the SON although the neural elements possessing the receptors have not been defined (Clark et al, 1986; Mansour et al, 1986; Tempel and Zukin, 1987; Mansour et al, 1988;) and the terminals of these neurones in the posterior pituitary do possess functional κ-opioid receptors (Zhao et al, 1988b). Our own work has confirmed the presence of μ- and κ-opioid receptors in the SON (Sumner et al, 1990; see Chapter 5) but no breakdown of these subtypes to reveal the presence of further putative subtypes was attempted. There is a growing literature describing these putative subtypes of κ-opioid receptor (Zukin et al, 1988; Traynor, 1989; Clark et al, 1989; De Costa et al, 1989; Rothman et al, 1989; Rothman et al, 1990). These studies of subtypes of opioid receptors present in the SON might be extended by creating suitable binding conditions with irreversible ligands and selective ligands to the putative subtypes of κ- and the μ-opioid receptors to determine whether these subtypes of receptor are present. Assigning function to these binding sites to provide an excitatory and an inhibitory opiate κ- or μ-opioid receptor might prove more difficult.

The presence of putative subtypes of κ- and μ-opioid receptors would also assist in

explaining the biphasic response to opiates by an indirect effect on an inhibitory input. If one subtype of κ-receptor was located presynaptically on an inhibitory terminal and the other post-synaptically on a magnocellular neurone, then it is conceivable that if the presynaptic receptor had a higher affinity and was therefore activated at a low dose of U50,488H (below the threshold for activation of the post-synaptic receptor) this would result in excitation of the postsynaptic element by disinhibition. At higher doses of U50,488H, the lower affinity postsynaptic receptor might be activated and mediate a direct inhibition of the soma so profound as to overcome the excitatory response to disinhibition. This hypothesis would be best investigated in vitro under conditions of synaptic blockade with a high Mg^{2+} medium, which if the hypothesis is accurate, should eliminate the excitatory phase of the biphasic response to U50,488H. If however, this is a direct effect through postsynaptically located receptors as in dorsal root ganglion cells, then synaptic blockade would presumably only remove any inhibitory and excitatory tone to these neurones and the acute effects of applied U50,488H would be unimpaired. An intracellular study in rat locus coeruleus with U50,488H where no low dose excitation was seen, does not disprove the presence of the κ-type of opioid receptor postsynaptically although it suggests that it is most likely to be located presynaptically (McFadzean et al, 1987). Further work to locate more precisely the subtypes of opiate receptor that might exist in the SON would take this area of discussion beyond speculation where for the moment it remains.

Because the low dose excitation with U50,488H was seen equally often in neurones from morphine-naive and morphine-tolerant rats, the phenomenon cannot be associated with the tolerant/dependent state exclusively and the mechanism of low dose excitation seems to be an acute opiate effect mediated at κ- and the μ-type opiate receptors (see Chapter 1).

The fact that this low dose excitation by U50,488H was meaningful in terms of plasma OT concentration suggests its importance. Although the dose of 1μg/kg i.v. did not elevate plasma OT, the next dose of 10μg/kg did so significantly (P=1.2x10-5,

Wilcoxon test for independent samples). These two doses were used as they constituted the range of threshold excitatory dose of U50,488H in terms of electrical activity of the OT-secreting neurones in the SON. It is not surprising that the lowest dose of 1μg/kg was not effective at raising plasma OT concentration as it was excitatory only in a minority of neurones tested and the plasma response reflects the combined activity of all OT-secreting neurones in the SON and PVN. In this group it was noticable that whilst 3/8 cells were excited by U50,488H at between 1-10μg/kg i.v. (1 at 1μg/kg and 2 at 10μg/kg) 5/8 cells were significantly inhibited in this dose range (2 at 1μg/kg and 3 at 10μg/kg) so the lack of plasma response at 1μg/kg is not surprising as the electrical response is mixed. Given this, it was somewhat unexpected that the plasma response to 10μg/kg U50,488H was so marked (see Figure 2.3.4.1.) as again it was excitatory in only half of the neurones in the electrophysiological study.

At 10μg/kg U50,488H the activity of the 2 cells excited was increased to a mean of 117.0 ± 0.13% control firing rate, whilst the activity of the 3 cells inhibited at 10μg/kg was reduced to 65.1 ± 17.1% control firing rate. This reflects an equivocal response in terms of the overall activity in the SON after U50,488H at 10μg/kg and does not tally with the plasma response, as plasma OT was raised to 42.1 ± 5.7 pg/ml, that is to 475.8% control plasma OT concentration (P=1.2x10-5, significantly different from the control plasma OT concentration, Wilcoxon test for independent samples; control is the mean of plasma samples 1 an 2). This could be accounted for in two ways: firstly, the sample of neurones randomnly selected might simply be unrepresentative of the full spectrum of activity and responsiveness of OT-secreting neurones in the SON and PVN or secondly, the effect of increasing firing rate from a basal level might be a more potent influence on OT secretion than inhibition of activity by a similar degree is on reduction in plasma OT.

The latter suggestion is supported by the plasma response to a dose of U50,488H of 1mg/kg i.v. which in terms of electrical activity is profoundly inhibitory to these neurones: of 4 cells tested at this dose in the vehicle-infused group, 1 cell was not

inhibited and remained unaffected at 5mg/kg. The 3 cells inhibited were inhibited to 12.6 ± 1.2% control firing rate. However, plasma OT in the control group which received 1mg/kg U50,488H was 10.4 ± 1.3pg/ml and this was not significantly different from the control value, derived from plasma samples 1 and 2, 10.2 ± 0.2pg/ml (Wilcoxon test for independent samples). This contrasts with the effect of the same dose of U50,488H given to the U50-treated group, after which plasma OT concentration was 15.1 ± 1.7pg/ml which is still significantly different from the plasma value for the control group at this dose (P=1.9x10-3, Wilcoxon test for independent samples). Presumably this dose of U50,488H, so powerfully inhibitory to the cell bodies of these neurones in the SON, is antagonising the excitatory effect of the previous dose, which might explain why the plasma OT concentration remains slightly elevated compared to the control group. Unfortunately there is no data on how long the increase in plasma OT would be expected to last for after 10μg/kg U50,488H as the protocol in the electrophysiological study precluded an assessment of the duration of effect of this dose beyond 10min and the blood sampling study did not contain a time control to investigate this aspect. U50,488H would be expected to persist from the previous injection however and therefore to possibly be maintaining OT secretion at an elevated level. So perhaps U50,488H at 1mg/kg inhibits electrical activity and OT secretion most effectively if it is already stimulated but is impotent to further depress OT release from basal levels. Insertion of a time control at this point in the protocol would have been helpful in resolving this point.

So these data suggest that the excitatory electrical response to U50,488H more potently influences OT secretion than does the inhibitory electrical response under basal conditions. Of course this conclusion assumes that OT-secreting neurones in the other major nucleus, the PVN, behave similarly and this could be investigated.

The effect of electrical activity on OT release has been studied (Bicknell, 1988) and it seems that frequency-dependent modulation of OT release occurs such that the terminals of OT neurones in the neurohypophysis release OT more efficiently as

frequency increases: 'frequency facilitation'. This phenomenon might help to illuminate the observed disproportionate effectiveness of increasing firing rate on OT release that is demonstrated by the electrophysiological and blood sampling studies.

The effect of naloxone on plasma OT concentration in the blood sampling study is also interesting. The plasma OT concentration in the control group as expected, did not change after isotonic saline injection (high dose of U50,488H only). The low and high dose U50,488H-treated group received naloxone (5mg/kg) however and this was followed by a rise in plasma OT to 1134% of its basal control value ($P=1.2x10-5$, Wilcoxon test for independent samples, against its control and $P=7.7x10-4$, Wilcoxon test for independent samples against equivalent control group sample). This can be accounted for by naloxone-reversal of endogenous opioid inhibition of OT secretion known to occur at the neurohypophysis under urethane anaesthesia in otherwise normal conditions (Sumner et al, 1989). It does not result from excitation of cell bodies in the SON as there was no evidence for this in the electrophysiological study in i.c.v. vehicle-infused rats. In these experiments naloxone restored firing rate to 90.3 ± 14.8% control firing rate with no evidence of overshoot in activity indicating no dependence upon U50,488H given acutely or upon any tonically active endogenous opioid acting at the level of the cell bodies in the SON.

2.4.3. Effects of i.v. U50,488H on supraoptic putative oxytocin neurones from i.c.v. morphine-infused rats

There was no evidence of tolerance to i.v. U50,488H after chronic i.c.v. morphine infusion. I conclude from these data (see Figure 2.3.5.3. for comparison of acute U50, 488H and acute morphine effects on OT neurones in chronic i.c.v. morphine-infused rats) that tolerance to morphine cannot be conferred to U50,488H in this neuroendocrine system.

Cross-tolerance occurs when the development of tolerance to one opioid is accompanied by tolerance to another opioid. Where tolerance and dependence occur together as in OT-secreting neurones, the following definition of cross-dependence is

helpful: "the ability of one drug to suppress the manifestations of physical dependence produced by another and to maintain the physical dependent state...this can be partial or complete and the degree is more closely related to pharmacological effects than to chemical similarities...in general, any potent morphine-like opioid will show cross depndence with other opioids that act on the same receptors" (Jaffe, 1985). It is on this basis that methadone is used in "substitution treatment" for morphine addiction as it also acts on the μ-opioid receptor and can prevent the onset of the morphine withdrawal syndrome. Methadone does have a withdrawal syndrome of its own but because it is longer-acting than morphine it produces a longer-lasting but much less intense withdrawal syndrome - the rationale behind its use. Generally, the development of cross-tolerance suggests strongly that the two opioids are acting at the same type of receptor (Wuster et al, 1983) or that the receptors interact to affect each others avidity for its ligand.

In rat brain membranes in vitro which were acutely exposed to μ- and δ-receptor ligands, there is evidence of a non-competitive interaction between the two binding sites and the authors of this study propose an opioid receptor complex with both μ- and δ-receptors which can interact thus allowing the receptor complex to activate intracellular mechanisms differentially according to the nature of the ligand(s) bound (Rothman et al, 1985). Morphine-dependent mice are cross-tolerant to the pupillary effects of Met-enkephalin and DADLE, both of which act at the δ-opioid receptor primarily (Ben-am and Korczyn, 1986). Similarly, there is cross-tolerance between the μ-opioid receptor agonist, morphiceptin and DPDPE, a highly selective δ-opioid receptor agonist acting at the rat spinal cord to produce analgesia (Russell et al, 1986). These findings are opposed in another study, this time in mice, where i.c.v. morphine does not confer cross-tolerance to DPDPE in terms of analgesia (Heyman et al, 1986). This may be due to species variation as other studies in mice are in agreement with this study. Cross-tolerance does not develop between the μ- and the δ-opioid receptors in mouse vas deferens when the μ-receptors are chronically stimulated in vivo with

morphine sulphate and then challenged in vitro with Leu-enkephalin which interacts with the δ-opioid receptor (Illes et al, 1980). Nor does cross-tolerance develop between sufentanyl (μ-receptor agonist) and D-Ala^2, D-Leu^5-enkephalin (δ-receptor agonist) in the same preparation (Schulz et al, 1980).

It seems that in the rat at least, there is some overlap in the characteristics of the δ- and μ-opioid receptors and their physiological roles. Perhaps species differences, dissimilar protocols and models and the use of different "selective" ligands in the various studies has resulted in a confusing picture concerning this point.

In the isolated mouse vas deferens taken from chronically opiate-treated mice, cross-tolerance was investigated between the μ-, κ- and the δ-opioid receptor agonists, sufentanyl, ethylketazocine and DADLE, respectively (Wuster et al, 1981). Preparations made tolerant to the μ-opioid receptor agonist, sufentanyl, showed no signs of cross-tolerance to other receptor subtype-selective ligands and similar results were obtained using preparations rendered tolerant to the κ- and the δ-opioid receptor ligands. Similar results were obtained in isolated guinea pig ileum and in intact animals (Schulz et al, 1981). So cross-tolerance does not always occur between opioids. If the three accepted subtypes of opioid receptor shared a common transduction system in the membrane, then one would expect tolerance in one receptor subtype to result in tolerance to the other two subtypes which had not themselves been chronically exposed to their selective ligands. This did not occur and therefore the subtypes of receptor are distinct in this respect. In this system, there was likewise no evidence of cross-tolerance: the two receptor subtypes under study appear to use independent transduction mechanisms.

There is however other recent evidence pointing to some degree of interaction between the μ- and the κ-opioid receptor subtypes. The chronic coadministration of U50,488H with morphine blocks the expected development of tolerance to morphine analgesia in the rat. The loss of morphine analgesia consequent to the development of tolerance to morphine is reversed transiently by the acute administration of U50,488H

but morphine analgesia induced by acute morphine administration is not affected by acute U50,488H (Yamamoto et al, 1988). There is clearly then some interaction between the μ-opioid agonist morphine and the κ-opioid agonist U50,488H in terms of tolerance to analgesia but exactly what this constitutes is not known. The previous report proposes that there is a receptor interaction and supports this hypothesis with similar experimental results in mice (Tulunay et al, 1981; Schmauss and Herz, 1987) and binding studies which have demonstrated interactions between μ-opioid binding sites and κ-opioid binding sites by a non-competitive mechanism (Garzon et al, 1982). Other reports observe cross-tolerance between a μ-selective and a κ-selective ligand in rats (Petrie et al, 1982; Hong et al, 1986). The "selective" κ-opioid receptor ligands in these studies were ethylketocyclazocine and dynorphin(1-13) neither of which act exclusively at the κ-receptor (Kosterlitz and Paterson, 1985) which implicates the possibility of action at other opioid receptors in the development of cross-tolerance. In fact there are many reports describing lack of cross-tolerance development between μ-selective agonists and κ-selective agonists in rats (Young and Khazan, 1984; Barr et al, 1986; Bhargava et al, 1989) in mice (Mansour and Valenstein, 1986; Solomon et al, 1988) guinea pigs (Schulz et al, 1981a; Schulz et al 1981b; Vaught, 1981; Von Voightlander and Lewis, 1988) and pigeons (Craft et al, 1989). The problem of ligand selectivity in investigating this phenomenon was highlighted by Von Voightlander and Lewis who demonsrated cross tolerance between morphine and ethylketocyclazocine and ketazocine but no cross-tolerance to the highly κ-selective agonist, U50,488H (VonVoightlander and Lewis, 1982).

Given these scenarios for the μ- and the δ-receptors and μ- and κ-receptors, the discovery of cross-tolerance between morphine and U50,488H in this neurosecretory system was conceivable. However, it seems that in OT-secreting neurones of the SON, the two receptor types are sufficiently distinct to allow no mutual interaction during the process of the development of tolerance to morphine at the μ-receptor. I have not investigated whether there is any acute interaction.

As stated earlier, cross-tolerance usually infers some overlap in the recognition process between different receptor types for their ligands and μ- and κ-opioid receptors are distinguished very well in other systems: the basis for their separate classification originally according to the binding of selective ligands (Robson et al, 1980) and to the selective development of tolerance (Wuster et al, 1983). So the present findings accord with the concensus in the literature.

One could speculate that there might be tolerance to endogenous opioids acting at the μ-opioid receptor with no effect on endogenous ligands acting at the κ-opioid receptor such that upon opiate withdrawal, precipitated by naloxone, the removal of opiate restraint on the OT-secreting system might be superimposed upon a similar withdrawal mediated through the endogenous opioid system. This could be investigated by in situ hybridisation of the endogenous opioid mRNA before and after the onset of tolerance and after precipitated withdrawal from morphine to assess adaptation in endogenous opioid systems. There is experimental support for this scenario. Chronic exposure to morphine has resulted in cross-tolerance to the endogenously produced opioids, Met-enkephalin, Leu-enkephalin and β-endorphin (Sivam and Ho, 1984) and physiological stimulation thought to activate the enhanced release of endogenous opioids results in the development of tolerance to exogenous morphine in mice with withdrawal-like behaviour (Christie et al, 1982). This possibility raises the question of tolerance to and dependence upon endogenous opioid peptides occurring under certain physiological conditions when opioid restraint is undesirable or when there is a need to abruptly produce a hyperresponsiveness in a system normally under opioid restraint. This has been postulated to be of importance in the OT-secreting system during the build up to and at the time of parturition in the rat when OT release must be tightly controlled and finally substantially increased (Jones and Summerlee, 1986; Bicknell et al, 1988a).

The low dose excitation seen in the i.c.v. vehicle-infused group was also seen in the i.c.v. morphine-infused group, in half of cells tested at 1-10μg/kg U50,488H. This

phenomenon cannot be associated with the development of tolerance uniquely as it is seen equally often in vehicle-infused rats.

There is some evidence for the binding of the κ-opioid selective ligand U50,488H to the μ-opioid receptor under suitable conditions when it can act as a receptor antagonist (Gillan et al, 1981; Corbett and Kosterlitz, 1986). One consequence in this protocol could be some reversal of morphine dependence by acute U50,488H with a resultant degree of excitation, seen only in full after the administration of the opiate antagonist naloxone. This seems unlikely as the effect is observed only at low doses and not at higher doses. The effect would be expected to increase in degree with increasing dose if it was explained simply by antagonism of morphine dependence at the μ-opioid receptor. So, the possible explanations above to account for low dose excitation also apply to the same phenomenon in the i.c.v. morphine-treated group and this suggests that this mechanism is not affected by adaptive changes that constitute tolerance to morphine in this neurosecretory system.

Naloxone produced withdrawal excitation similar to that described in the previous Chapter and this confirms dependence. It can also be assumed that tolerance to morphine would have been present in these experiments although this was not tested: the protocol was identical to that in Chapter 1 in which tolerance was demonstrated to morphine, apart from the acute administration of U50,488H instead of morphine and the range of activity of non-phasic neurones in these experiments was similar to control. The acute administration of U50,488H does not appear to have impaired or enhanced the expression of the withdrawal response.

In conclusion then, tolerance is not conferred to the κ-opioid receptor-selective agonist, U50,488H after chronic activation of the μ-opioid receptor by the selective agonist morphine in the OT neurosecretory system.

CHAPTER 3

DOES ACUTE MORPHINE ACTION ON OXYTOCIN NEUROSECRETORY NEURONES IN VIVO INVOLVE A PERTUSSIS TOXIN-SENSITIVE G-PROTEIN?

3.1. INTRODUCTION

The acute effects of opiates are mediated through opiate receptors of which there are three widely recognised subtypes, μ, κ and δ, as defined by the differential affinity with which highly selective ligands bind to them (Gilbert and Martin, 1976; Lord et al, 1977). Activation of neuronal opiate receptors has been reported to lead to a variety of cellular events including the inhibition of adenylate cyclase (AC) activity (Collier and Roy, 1974; Sharma et al, 1975a; Sharma et al, 1975b; Klee and Nirenberg, 1976; Lampert et al, 1976; Goldstein et al, 1977); increase in a membrane potassium conductance resulting in membrane hyperpolarisation and causing a reduction in spontaneous activty or a reduction in the amount of neurotransmitter released (for a review see, North, 1986); changes in a membrane calcium conductance, again potentially leading to a decrease in the level of spontaneous activity and to a reduction in neurotransmitter release, by agonists at the μ-type opiate receptor acting by an indirect action on the conductance (Tokimasa et al, 1981; North and Williams, 1985) or via the κ-type opiate receptor by a direct action (Werz and Macdonald, 1984a; Werz and Macdonald, 1984b; Werz and Macdonald, 1985) effects upon the activity of cell phosphorylases and phosphatases which regulate enzymes, hormone/neurotransmitter receptors and ion channels (Clark et al, 1972; O'Callaghan et al, 1982).

A role has been defined in the last decade for a family of intramembrane GTP-binding proteins (G-proteins), as transducing elements between a variety of receptor molecules and their effector systems, such as AC (Bokoch et al, 1984; Neer et al, 1984; Sternweis and Robishaw, 1984; Gilman, 1986; Sternweis and Pang, 1990). G-proteins have been implicated in the control of a range of intracellular second messenger systems, including AC (Stryer and Bourne, 1986; Gilman, 1987), phospholipase C (Cockcroft and Gomperts, 1985), cGMP phosphodiesterase (Stryer et al, 1981) as well as ion channel proteins (Pfaffinger et al, 1985; Holz et al, 1986).

They can mediate either inhibitory or excitatory events within a cell depending on the type of G-protein and associated receptor. The stimulatory G-protein, Gs is selectively acted upon by cholera toxin which ADP-ribosylates the GTP binding site, enhancing its activity (Gill and Meren, 1978) and cellular events triggered by the activation of inhibitory neurotransmitter receptors have been associated with the 'inhibitory G-protein', Gi (Milligan et al, 1986). Pertussis toxin (PT) deactivates Gi by binding to the GTP-binding site and irreversibly ADP-ribosylating, thus preventing the exchange of GDP for GTP which normally takes place during its activation (Sullivan et al, 1987). Because it does not likewise affect Gs, PT differentiates between Gi and Gs. However, PT also deactivates another G-protein found in abundance in neuronal tissue, Go (Sternweis and Robishaw, 1984).

The overlap in cellular events affected by either opiate receptor activation or by G-protein activation points to a possible connection between the two.

Acute opiate effects can be impaired by PT treatment. (Abood et al, 1985; Aghajanian and Wang, 1986; Crain et al, 1987; North et al, 1987; Duman et al, 1988; Parolaro et al, 1990).

These observations implicate a PT-sensitive G-protein, Gi or Go in post-receptor transduction for opiates. Pursuing this possibility further, other studies have identified chronic opiate effects on the dynamics of inhibitory G-proteins (Lang and Schulz, 1989; Nestler et al, 1989). These findings corroborate the concensus to date, that an inhibitory PT-sensitive G-protein is involved in post-opiate receptor transduction.

So, of particular interest is the potential role these G-proteins might play in the physiological adaptation that occurs in some cells when exposed chronically to opiates - tolerance with associated dependence, given that in oxytocin-secreting cells of the SON, tolerance develops to chronic i.c.v. morphine in terms of firing rate (see Chapter 1). The possibility arises that this adaptation involves regulation of an associated G-protein. It has yet to be shown however that opiate receptors associated with these cells or with neurones which project to the SON are linked to a PT-sensitive G-protein

at all and the primary aim of the present study was to investigate this question.

A protocol similar to that already used earlier (see Chapters 1 and 2) was used to test the sensitivity of these neurones to the μ-selective agonist, morphine and to the κ-selective agonist, U50,488H given acutely by i.v. injection after pretreatment with i.c.v. 0.9% saline in the control group or after i.c.v. PT in the treated group. Thus it was hoped also to describe any difference between the μ- and κ-opiate receptors responsible for mediating the action of i.v. opiates on SON neurones in terms of their association with G-proteins.

3.2. METHODS

3.2.1. Animals

Virgin female Sprague Dawley rats (200-250g; Bantin and Kingman) were housed at 21-23°C and allowed food (standard breeder diet) and tap water ad libitum; they were kept under a 13h/11h light/dark cycle (on 08.00, off 21.00).

3.2.2. Intracerebroventricular injection of pertussis toxin or 0.9% saline

Purified pertussis toxin (lyophilised in phosphate buffered saline) was a gift from Dr J.H. Freer, Microbiology Dept., University of Glasgow. Three days before the day of experiment, rats were injected i.c.v. with 5μl 0.9% sterile pyrogen-free saline or with 7μg PT in 5ml 0.9% sterile pyrogen-free saline acutely as follows: under ether anaesthesia rats were placed in a stereotactic frame and the skin overlying the skull incised. The surface of the skull was cleared of tissue and bregma and lambda visualised and used as reference points to level the skull. A hole was then drilled at the coordinates 0.6mm caudal and 1.6mm lateral to bregma. The injectate was contained in a Hamilton 5μl 'through the needle' microsyringe (#7005; Hamilton) and was injected via a 600μm o.d. needle lowered to 4.5mm below the skull surface to enter a lateral cerebral ventricle. The incision was then sutured and the rat allowed to recover. Rats were weighed daily to monitor their well being (after initial greater weight loss than controls, the PT-injected rats recovered and appeared healthy on the day of experiment).

3.2.3. Electrophysiology

Rats were anaesthetised with urethane (ethyl carbamate, 1.25g/kg i.p.) and the trachea and an external jugular vein cannulated. The rat was mounted in a stereotactic frame and the right SON and the neural stalk exposed by ventral surgery (ventral surgery was performed by Dr G. Leng; Leng, 1980). A concentric bipolar stimulating electrode was placed on the pituitary stalk to deliver pulses (1ms matched biphasic pulses <1mA peak to peak, 0.3Hz) designed to antidromically activate neurones

projecting from the SON to the posterior pituitary. A glass micropipette filled with 0.9% saline (20-40MOhm) was placed under visual control into the SON to record the activity of antidromically-activated supraoptic neurones. The criteria used to select only putative OT neurones in the SON described in Chapter 1 also applied here.

Recordings were made and stored on videotape in digital format via a modified pulse code modulator for later analysis. Online analysis was performed using software written for this application (R. Bunting, Dept. Neuroendocrinology, AFRC Institute of Animal Physiology and Genetics Research, Babraham, Cambridge; see Chapter 1) and the interval between consecutive spikes measured and averaged in 30 second bins for the construction of histograms. Firing rate was recorded for at least 10 minutes to establish a stable control period and to identify the cell, then morphine sulphate B.P.(Pharmacy, Royal Infirmary of Edinburgh) or U50,488H (Upjohn Ltd. or Sigma Chemical Co.) both in 0.9% saline were injected i.v. in increasing dose: 1, 10, 50, 100, 500, 1000 and 5000μg/kg in an injection volume of 500μl/kg. When the end of the protocol was reached or when a maximal inhibition was achieved, naloxone HCl was injected i.v. (5mg/kg; Sigma Chemical Co.).

The criteria for experimenting upon cells newly recorded if the original cell was lost after the drug protocol had already started, are outlined in Chapter 1. Suffice to say the history of the population of neurones in each rat was always carefully considered (as before, see Chapter 1) when interpreting the results obtained from an individual experiment.

Separation of the 95% confidence bands between the control period and the drug treatment period was used as a measure of the significance of a response.

3.2.4. Statistics

Comparison was made of the threshold cumulative inhibitory dose of morphine or U50,488H between treatment groups using the Wilcoxon test for independent samples. Comparisons were also made of the degree of inhibition at given doses of morphine or U50,488H by comparison of mean firing rate after drug, again between treatment

groups. Comparison was also made where possible of threshold voltage and latency to antidromic activation. Where sufficient data on electrophysiological properties (before drug injection) were unavailable for analysis for the control i.c.v. 0.9% saline-injected group, data from an untreated control group was used as indicated in the Results section of this Chapter.

3.3 RESULTS

3.3.1. I.c.v. 0.9% saline-injected control group

3.3.1.1. General activity observed

In this group information was not sought from cells that were not tested and details of only one untested cell were noted; this was a phasic cell with a latency to antidromic activation of 16.6ms and a threshold voltage to antidromic activation of 34V. Of the 6 cells tested, 5 were continuous (mean firing rate 2.7 ± 0.3 Hz), firing at more than 2 Hz. One cell was classed as slow-irregular, firing at 1.96 Hz.

3.3.1.2 Characteristics of non-phasic putative oxytocin neurosecretory neurones

For non-phasic neurones (ie. continuous and slow-irregular), mean ± SE firing rate of active neurones was 2.6 ± 0.3 Hz (range 1.96 - 4.0 Hz; 1<2 Hz; n=6 cells, 5 rats). Mean ± SE latency to antidromic activation was 17.9 ± 1.5 ms (n=5 cells, 5 rats). Mean ± SE threshold voltage to antidromic activation was 35.6 ± 17.0 V (n=5 cells, 5 rats).

3.3.1.3. Effects of i.v. morphine on non-phasic putative oxytocin supraoptic neurones in i.c.v. 0.9% saline-injected rats

Acute doses of morphine and U50,488H are expressed as cumulative doses and this is justified in Chapters 1 and 2.

Both inhibitory and excitatory effects of morphine were seen. The effect of morphine was evident within 30 seconds of injection.

The cumulative threshold dose of morphine to significantly inhibit the 5/6 cells inhibited by morphine was between 11 and 261μg/kg (median cumulative threshold inhibitory dose: 61μg/kg). At a cumulative dose of morphine of 161μg/kg, 5 cells tested were inhibited significantly to 25.7 ± 11.7% control firing rate (CFR; see Figure 3.3.1.4.). The mean firing rate of the 5 neurones that were inhibited by morphine was 2.3 ± 0.1 Hz (range 1.96 to 2.46 Hz). The CFR of the cell that did not respond to morphine at this dose was 4.0 Hz. At the higher cumulative dose of 661μg/kg

morphine, 6 cells were inhibited to 4.85 ± 1.0% CFR. At the highest doses used, at or above 161μg/kg, (between 161 and 761μg/kg) firing rate was reduced to a mean of 4.4% (n=5).

At 1μg/kg morphine, 2 of 5 cells tested were excited significantly, 3 were unaffected and none were inhibited. At 10μg/kg morphine, 2 of 5 cells tested were excited significantly, 2 were unaffected and 2 were inhibited significantly. The 2 cells excited at 1μg/kg were also excited at 10μg/kg morphine. They were both inhibited by higher doses of morphine. None of these cells was tested with U50,488H, since they were strongly inhibited by morphine.

In those cells tested with naloxone HCl (5mg/kg), firing rate was restored to 116.5 ± 14.3 % CFR (n=4).

Figure 3.3.1.4.(overleaf) Pertussis toxin control group: summary of firing rate changes

Each histogram is the record of a single neurone in one rat which had received a single i.c.v. injection of 5μl 0.9% sterile pyrogen-free saline 72hours prior to the day of experiment. Rats were anaesthetised with urethane and a supraoptic nucleus exposed for electrophysiological recording from putative oxytocin neurones. Morphine (M) was given i.v. in increasing dose and firing rate collected in 30sec bins for calculation of 10 minute mean firing rate. A significant change in firing rate was denoted by separation of 95% confidence bands about the control firing rate and those of any drug treatment period. A significant difference is marked with an asterisk. At low doses, morphine was sometimes excitatory (see cells b) and c)) but at higher doses was inhibitory.

The control period firing rates were a) 2.4Hz b) 2.0Hz c) 2.4Hz d) 2.5Hz e) 4.0Hz and f) 2.2Hz.

Naloxone (NAL; 5mg/kg i.v.) where given reversed this inhibition.

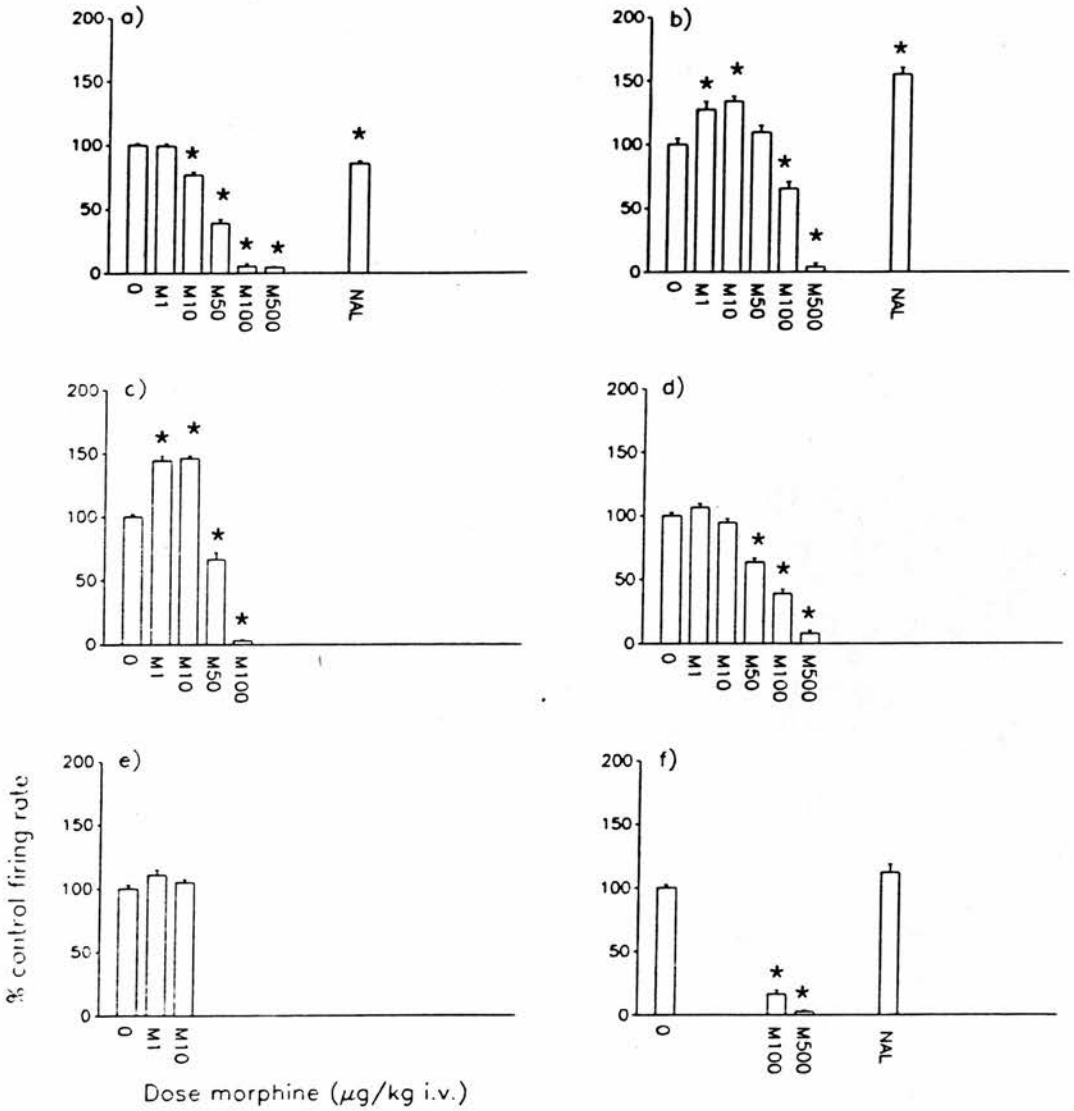

a)
b)
c)
d)
e)
f)
200
150
100
50
0
0
M1
M10
M50
M100
M500
NAL
% control firing rate
Dose morphine (μg/kg i.v.)

Figure 3.3.1.4.

3.3.2. I.c.v. pertussis toxin (PT)-injected group

3.3.2.1 General activity observed

As a large set of data about the range and types of activity observed in the control, i.c.v. saline-injected group was not collected, comparison was made between the untreated control group of Chapter 1 which underwent a similar protocol, and the i.c.v. PT-treated group of this series. The assumption of equivalence between the i.c.v. saline-injected group and the untreated control group is supported by comparison of the available data common to the two groups - i.e. from the i.v. morphine-tested cells, a comparison of CFR, before the application of drugs. In addition latency to antidromic activation and threshold voltage to antidromic activation were observed, although the latter two variables were not of primary interest. For firing rate, latency and threshold voltage, in no instance did comparison between the untreated control group and the i.c.v. 0.9% saline control group achieve statistical significance (significance level 95%, allowance made for 3 comparisons, Wilcoxon).

In 10 i.c.v. PT-treated rats, control period recordings were made from 30 cells. 14 (46.7%) were continuous (not significantly different vs. untreated control group), firing at more than 2Hz. 5 (16.7%) fired at less than 2Hz and were classed as slow-irregular cells (not significantly different vs. untreated control group). 6 (20.0%) fired phasically (not significantly different vs. untreated control group) and 4 (13.3%) were silent (not significantly different vs. untreated control group). In all the above tests, allowance was made for multiple comparisons and a level of significance of 95% was chosen.

3.3.2.2 Characteristics of non-phasic putative oxytocin neurosecretory neurones

For non-phasic cells (i.e. continuous and slow-irregular), mean±SE firing rate of active non-phasic cells (including slow cells) was 4.7±0.7 Hz (range 0.26-8.20, 2<1Hz, 5<2Hz; n=19 cells, 10 rats; not significantly different vs untreated control group, Wilcoxon; not significantly different vs i.c.v. saline-injected control group, Wilcoxon). Mean±SE latency to antidromic activation was 12.2±1.0 ms (n=16 cells,

10 rats; not significantly different vs control group, Wilcoxon; not significantly different vs i.c.v. saline-injected control group, Wilcoxon). Threshold voltage to antidromic activation could not be measured in this group due to lack of the necessary apparatus. In all statistical tests, allowance was made for multiple comparisons and where necessary a level of significance of 95% chosen.

3.3.2.3 Characteristics of phasic putative vasopressin neurosecretory neurones

The mean±SE CFR was 6.1±3.2 Hz (range 1.42-15.42, 0<1Hz, 1<2Hz; n=4 cells, 2 rats; not significantly different vs untreated control group). Mean ± SE latency to antidromic activation was 14±1.0 ms (n=6 cells, 2 rats; not significantly different vs control group).

3.3.2.4 Effects of i.v. morphine or U50,488H on non-phasic putative oxytocin supraoptic neurones in i.c.v. pertussis toxin-treated rats

Both inhibitory and excitatory effects of morphine were seen. The effect of morphine was always evident within 30 seconds of injection.

The cumulative threshold dose of morphine to significantly inhibit the 7/8 cells which responded was between 1 and 1661μg/kg morphine, median cumulative threshold inhibitory dose was 11μg/kg (not significantly different vs i.c.v. saline-injected control group, Wilcoxon).

At a cumulative dose of morphine of 161μg/kg, the firing rate of 6 cells tested was reduced following morphine to a mean of 89.29 ± 11.8% CFR (1 neurone from these 6 was not inhibited at this dose). Compare this with that for the control group: all 5 neurones were significantly inhibited at 161μg/kg morphine to a mean of 25.7 ± 11.7% CFR (P=0.014, Wilcoxon). The mean firing rate of the 5 control neurones which were inhibited by morphine at 161μg/kg in the i.c.v. PT-treated group was 4.6 ± 0.6 Hz, (range 3.08-5.85 Hz) which was significantly higher than that for the control group (P=0.042 vs i.c.v. saline-injected control group, n=5 (CFR control group: 2.3 ± 0.1 Hz) Wilcoxon). The CFR of the neurone that did not respond to morphine at 161μg/kg was 3.0 Hz.

At the higher cumulative dose of 661μg/kg morphine, 6 cells in the PT-treated group were tested (4/6 were inhibited significantly) and the mean firing rate of these 6 after morphine was reduced to 83.05 ± 19.4% CFR whereas in the control i.c.v. 0.9% saline-treated group, all 4 cells were inhibited to 4.85±1.0% CFR (P= 0.014, Wilcoxon). So, although the proportion of neurones affected at these two key doses were similar between groups, and the median threshold inhibitory dose was not different between groups, the degree of inhibition at a given dose of morphine, compared to the CFR was substantially greater in the i.c.v. 0.9% saline-treated group compared with the i.c.v. PT-treated group.

At the highest doses used, at or above 161μg/kg (between 161 and 6661μg/kg) firing rate was reduced to a mean of 50.3% (n=6). This compares with 4.4% in 5 control cells.

At 1μg/kg morphine, 3/7 cells tested were excited significantly, 3 were unaffected and one cell was inhibited. At 10μg/kg morphine, only one cell was excited and this cell was not inhibited by morphine up to 161μg/kg. Two of the remaining cells were unaffected whilst four cells were inhibited at this dose. This is a similar profile of excitation followed by inhibition to that seen in the chronic i.c.v. morphine-infused group described in Chapter 1, in both morphine-naive and chronic i.c.v. morphine-infused rats after acute i.v. U50,488H as described in Chapter 2 and to that seen in the i.c.v. saline-injected control group for this series.

Some cells were tested with both morphine and to U50,488H to assess any difference of PT treatment on drug effectiveness. The record of these cells is given in Figure 3.3.2.5.. In 4/5 cells tested with both drugs, U50,488H inhibited electrical activity more potently than morphine at either an equivalent or a higher dose. U50,488H was capable of profoundly inhibiting the spontaneous activity of cells only marginally affected by morphine (see Figure 3.3.2.5.). Of the 5 cells tested with U50,488H, 4 were inhibited maximally as follows (all doses are expressed as cumulative and a comparison is made, in brackets following, with the same cell's

response to an equivalent dose of morphine): at a dose of 50μg/kg U50,488H, to 87.8% CFR (cf to 137.1% after 61μg/kg morphine), at 500μg/kg U50,488H to 22.74% CFR (cf to 111.6% after 661μg/kg morphine), at 1.5mg/kg to 26.4% (cf to 74.4% after 1.661mg/kg morphine) and at 1mg/kg to 0.83% CFR (cf to 78.4% after 1.661mg/kg morphine). The maximum inhibition of firing rate achieved after morphine on the other hand, was to 117.1% CFR at 161μg/kg, 34.8% CFR at 6.661mg/kg, 68.3% CFR at 6.661mg/kg and 76.0% CFR at 6.661mg/kg respectively for the 4 cells described above, demonstrating a relative lack of effect of morphine upon the electrical activity of these cells.

In cells tested with naloxone HCl (5mg/kg), firing rate was restored to 80.8 ± 14.7 % CFR (n=4) compared to 117.4 ± 20.2 % (n=3) in the 0.9% i.c.v. saline-injected control group (P=0.133, not significantly different, Student's t-test for unpaired samples). So there is no evidence of withdrawal excitation in the PT-injected group.

Figure 3.3.2.5.(overleaf) Pertussis toxin-treated group: summary of firing rate changes

Each histogram is the record of a single neurone in one rat which had received a single i.c.v. injection of 7μg of pertussis toxin in 5μl 0.9% sterile pyrogen-free saline 72hours prior to the day of experiment. Rats were anaesthetised with urethane and a supraoptic nucleus exposed for electrophysiological recording from putative oxytocin neurones. Morphine or U50,488H (M or U) were given i.v. in increasing dose and firing rate collected in 30sec bins for calculation of 10 minute mean firing rate. A significant change in firing rate was denoted by separation of 95% confidence bands about the control firing rate and those of any drug treatment period. A significant difference is marked with an asterisk. At low doses, morphine and U50 were sometimes excitatory (see cells a), c), d), g) and h)) but at higher doses was inhibitory, although the potency of morphine (but not U50,488H) was much reduced compared with the i.c.v. saline control group.

The control period firing rates were a) 5.85Hz b) 5.45Hz c) 3.1Hz d) 3.4Hz e) 5.3Hz f) 6.5Hz g) 2.9Hz and h) 3.2Hz.

Naloxone (NAL; 5mg/kg i.v.) where given reversed this inhibition.

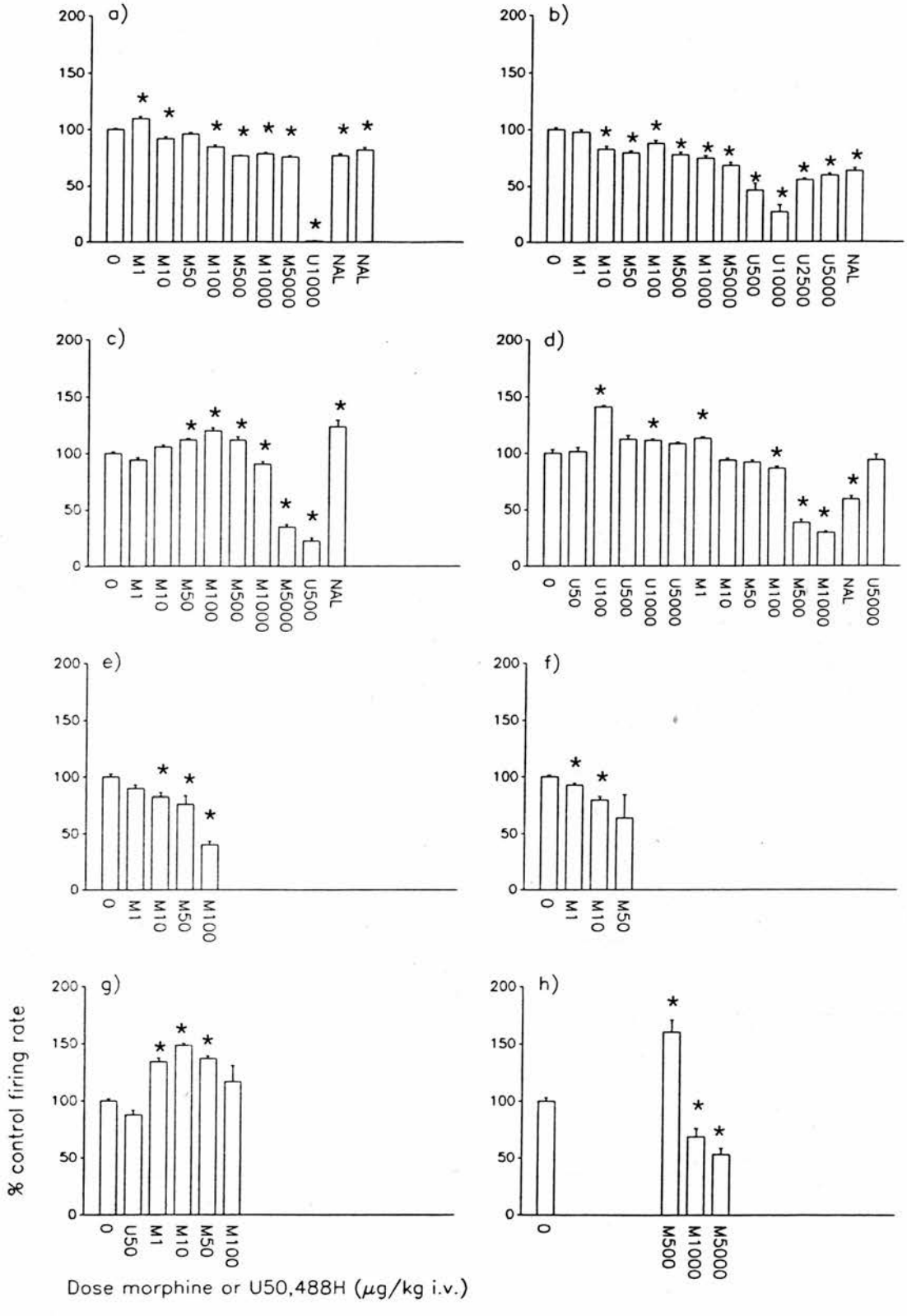

a)
b)
c)
d)
e)
f)
g)
h)
% control firing rate
Dose morphine or U50,488H (μg/kg i.v.)

Figure 3.3.2.5.

3.4. DISCUSSION

The results suggest that the i.c.v. injection of pertussis toxin (PT) 72h prior to testing with acute i.v. morphine impairs the powerful inhibitory action of morphine upon the electrical activity of these neurones under control conditions. However, this is not immediately apparent from a simple comparison of cumulative threshold dose to inhibition for morphine .

In previous Chapters, expression of the inhibitory action of morphine or U50,488H on identified oxytocin cells was in terms of the proportion of tested cells reaching threshold inhibition at each dose tested. This method was sufficient to accurately reflect the difference between control and chronic i.c.v. morphine-treated groups. This analysis when applied to neurones in the present series of experiments however, fails to faithfully describe the difference between the i.c.v. 0.9% saline-treated control group and the i.c.v. PT-treated group.

So, at a dose of 161μg/kg morphine 5 cells in the control group were significantly inhibited whereas 5/6 neurones in the PT-treated group were significantly inhibited. This expression is misleading since the depression in firing rate after morphine in those cells inhibited beyond threshold in the two groups was to 25.7 ± 11.7% CFR and 89.3 ± 11.8% respectively. Similarly, at the higher dose of 661μg/kg morphine, for the 4 cells tested at this dose in the i.c.v. 0.9% saline-treated group firing rate was reduced to 4.85 ± 1.0% CFR, all 4 cells being inhibited, but of the 6 cells tested at this dose in the PT-treated group the mean change in firing rate was an inhibition to 83.1 ± 19.4% CFR and of the 6 cells tested, two were excited at this dose but were inhibited significantly at the next dose of 1mg/kg.

At these two doses, significant differences arose in the degree of inhibition achieved by morphine in terms of spontaneous firing rate and I assume that these differences would have persisted had the control group been tested at the two highest doses of 1mg/kg and 5mg/kg. As it was, because maximal inhibition was achieved at

661μg/kg morphine, higher doses were not actually tested. These data are expressed in figure 3.4.1..

So, the inhibitory potency of morphine is impaired after i.c.v. PT treatment: this is likely to be the result of deactivation of a G-protein.

All G-proteins are constructed from 3 distinct components - they are heterotrimeric. The α-subunit is the largest at 39-52KDa and it differs between G-proteins but has a hydrophilic region which faces the inside of the cell membrane and has access to cytosolic GTP and GDP for which it has a binding site. PT catalyses the transfer of an ADP-ribose group onto a cysteine residue of the inhibitory G-protein Gi and this group then interacts with the GTP binding site thus inactivating the G-protein (Dolphin 1987; Dunlap et al 1987). The α-subunit also contains the recognition site for the effector molecules in the membrane and cytosol which it regulates, making the α-subunit the key regulatory component. The β- and γ-subunits are closely associated and hydrophobic and are embedded in the membrane. They are similar between G-proteins and are of lower molecular weight than the α-subunit. In the inactive state, the G-protein consists of these three components with GDP bound to the α-subunit. The interaction of agonist with receptor facilitates the exchange of GDP for GTP. Following the binding of GTP the G-protein dissociates into the α-subunit with inherent GTPase activity and the $\beta\gamma$-subunit. α-G-GTP can regulate the activity of a number of effector molecules such as adenylate cyclase (Stryer and Bourne, 1986; Gilman, 1987), phospholipase C (Cockcroft and Gomperts, 1985), cGMP phosphodiesterase (Stryer et al, 1981) and potassium and calcium channel gating molecules (Pfaffinger et al, 1985; Holz, et al, 1986). This effect is terminated by the hydrolysis of GTP and the reassociation of the α-subunit with free $\beta\gamma$- to form the inactive G-GDP (Dunlap et al, 1987).

The first G-protein to be described was Gs which is permanently activated by the catalytic transfer of an ADP-ribose group from NAD (nicotinamide adenine dinucleotide) to the GTP binding site on the α-subunit of the G-protein by cholera

toxin, an exotoxin from *V. cholerae* (Dolphin, 1987).

The toxin used in the present study, pertussis toxin, derived from *Bordetella pertussis*, ADP-ribosylates the GTP binding site of Gi, an inhibitory G-protein regulating the activity of second messenger molecules. As well as Gi, neuronal membranes contain large amounts of a third G-protein, Go, which can also be linked to AC and possibly to other second messenger systems. It has been implicated particularly in the regulation of calcium channel function in neurones (Hescheler et al, 1987). Both Gi and Go are substrates for PT.

I propose therefore that a Gi/o protein is coupled to the opiate receptor through which morphine acts in supraoptic neurones or their inputs to inhibit electrical activity. The i.c.v. application of PT impaired this coupling such that after the activation of the opiate receptor by morphine, a process not interfered with by PT, transduction via Gi/o does not occur as effectively as normal. Intracellular events that follow the activation of this Gi/o do not therefore occur to the same degree and the physiological response, inhibition of cell firing rate, is attenuated.

The fact that U50,488H more potently inhibited the firing rate of these cells (in 4/5 cells tested with both U50,488H and morphine) than did morphine at either an equivalent or even a higher dose demonstrates the striking contrast between the mechanism of action of these drugs. The potency of U50,488H appeared to be unimpaired when the effects are compared with those seen in the untreated control group in Chapter 2, whereas the effectiveness of morphine was very much impaired by PT treatment, so it can be concluded that U50,488H acting at κ-receptors on OT neurones does not act via a Gi/o protein.

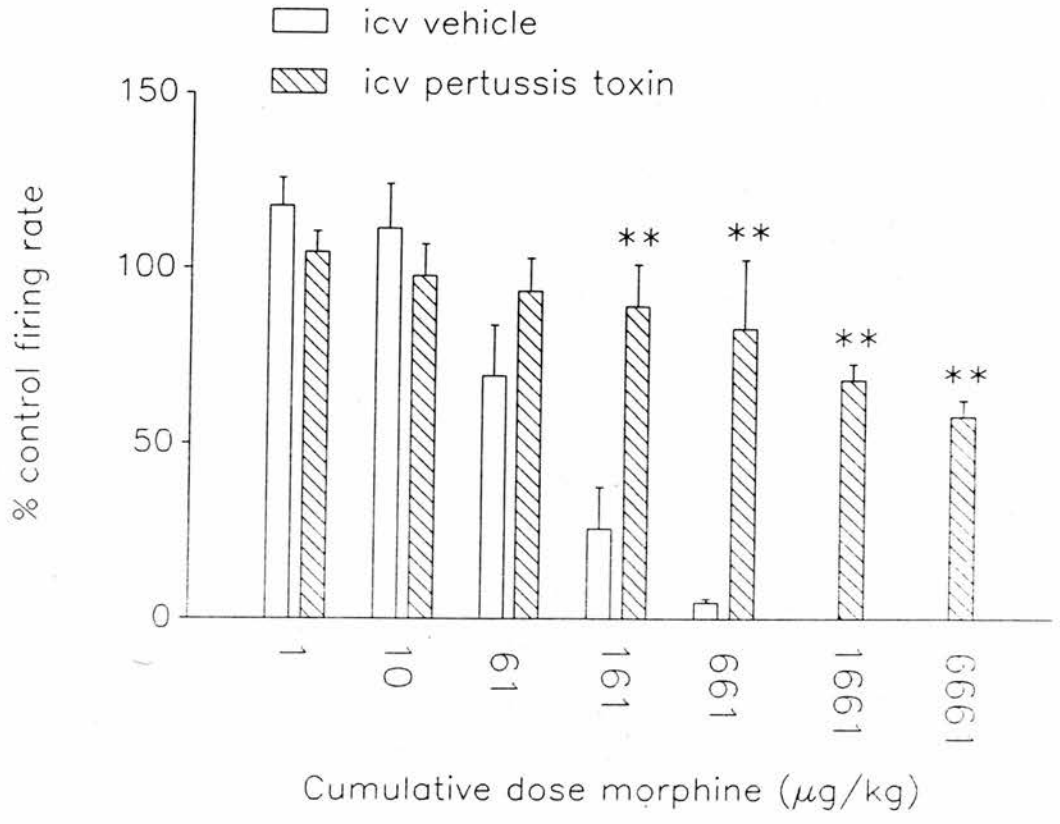


Figure 3.4.1. Comparative dose response relations for supraoptic putative oxytocin neurones from the i.c.v. vehicle-injected and the i.c.v. pertussis toxin-injected groups

Horizontal axis denotes the cumulative dose of morphine (μg/kg i.v.) and the vertical axis, the percentage of control firing rate achieved at each dose of morphine. The potency of morphine to inhibit neurones recorded in i.c.v. pertussis toxin-injected rats was much less than that in i.c.v. vehicle-injected rats and this difference was statistically significant at 161μg/kg morphine (Student's t-test).

3.4.2. Diversity in linkage between opioid receptors, G-protein and second messengers

μ-, δ- or κ-opioid receptors can be linked to a G-protein which may regulate a second messenger such as AC, or may be directly linked to an ion channel whose activity can be regulated. Both these results can be the result of agonist interaction with one of these receptors.

Various studies indicate that opioid receptors sensitive to morphine, probably μ-opioid receptors can be linked to G-proteins.

Rat locus coeruleus neurones are hyperpolarised by morphine and this effect is impaired by PT. Intracellular GTPγS, a non-hydrolysable form of GTP, restores the potency of morphine, indicating a role for a PT-sensitive GTP-binding protein (a G-protein) in the regulation of a hyperpolarising current in these cells (Wang and Aghajanian, 1987). This current has been described for agonists at opiate μ- and δ-receptors in guinea-pig locus coeruleus neurones as a potassium current and the G-protein confirmed as PT-sensitive, that is Gi/o and AC does not seem to be involved in channel regulation (Christie et al, 1987; North et al, 1987). Although AC is not involved in acute morphine effects in locus coeruleus neurones, AC is involved in the response to chronic morphine, as the activity of cAMP-dependent protein kinase is increased but cAMP-independent protein kinase activity is unchanged after chronic morphine (Nestler and Tallman, 1988). However, changes in cAMP do not seem to be acutely involved in the electrical response of neurones to opiates (Duggan and Griersmith, 1979; Karras and North, 1979), and likewise, the change in cAMP after chronic morphine described above need not be related to the electrophysiological consequences of morphine tolerance/dependence. Chronic but not acute morphine treatment increases levels of PT-induced ADP-ribosylation of α-subunits of Gi/o in rat locus coeruleus neurones in a naloxone-sensitive way (Nestler et al, 1989; see later Discussion).

Although DAGO ([D-Ala^2,N-Me-Phe^4,Gly^5-ol]-enkephalin) and the δ-receptor

ligand DPDPE ([D-Pen^2,D-Pen^5]-enkephalin) are both linked to an inhibitory G-protein, only DAGO inhibited AC in guinea-pig striatal membranes indicating that G-protein coupling to AC in these cells depends on the type of receptor (Satoh et al, 1990).

For guinea-pig myenteric plexus neurones, incubation of ileum with PT or pretreatment of guinea-pigs with PT intraperitoneally reduced the inhibition of cholinergic transmission seen with morphine, probably a result of the accompanying ADP-ribosylation of Gi and Go in myenteric neurones. The concentration-response curve for morphine was shifted to the right and the maximum response decreased. The degree of attenuation of the inhibitory potency of morphine coincided with the degree of ADP-ribosylation of Gi or Go which were not distinguishable (Johnson et al, 1990).

Rat caudate nucleus contains opioid receptors which are linked to AC through a G-protein (Law et al, 1981; Abood et al, 1985; Kamikubo et al, 1986).

In neuroblastoma x glioma (N x G) hybrid cells which possess only δ-opioid receptors, DADLE inhibited a voltage-dependent calcium current (Schultz et al, 1990). This effect was not mediated by a change in intracellular cAMP or cGMP or the phosphatidyl inositol system (McFadzean et al, 1989). PT treatment or treatment with antibodies to αi or αo (the α-subunits of the inhibitory G-proteins Gi and Go respectively) abolished this effect. Intracellular application of the purified α-subunit of either Gi or Go via a patch pipette to N x G cells pretreated with PT restored the inhibitory effect of DADLE, αo more potently than αi (Hescheler et al, 1990). These results were augmented by a study photolabelling the αi and the αo subunits of these cells as the opiate receptor was activated. For αo but not for αi there was a good correlation between the ability of agonists to inhibit the calcium current and the degree of photolabelling of the α-subunit, implicating endogenous Go but not Gi in the transduction of this opioid effect (Hescheler et al, 1990). However, another study has demonstrated a δ-opioid-induced inhibition of AC which is mediated by a G-protein

(Koski et al, 1982; Kurose et al, 1983). In conclusion, Gi/o is not always linked to AC as a post-opioid receptor mechanism.

3.4.3. Opioid receptors can be associated with the stimulatory G-protein, Gs

Adding to this already complex picture, recent work has shown that opioid receptors do not always associate with Gi/o but can be linked to Gs which then mediates 'excitatory' events in the neurone.

In dorsal root ganglion cells, opioid receptors mediate both "inhibitory" and "excitatory" events through Gi/o and Gs respectively (see Chapter 1, p56 for more detail). They are activated at different concentrations of opioid: the Gs protein positively couples the receptor to AC and cAMP-dependent voltage-sensitive ionic conductances whilst the Gi protein is activated at higher concentrations of opioids and activates voltage-sensitive ionic conductances probably independently of cAMP (Werz and Macdonald, 1985; North, 1986; Shen and Crain, 1989; Shen and Crain, 1990a).

3.4.4. G-proteins might not activate a conventional cytosolic second messenger system but might act on an ion channel protein directly

In addition to activation of AC and its regulatory action then on ion channels, the possibility exists, as shown first in cardiac muscle, that G-proteins can regulate potassium channels directly. Acetylcholine binds to a muscarinic receptor which is coupled to a G-protein and receptor activation increases the probability of potassium channel opening and second messengers such as cAMP, cGMP, diacylglycerol (DAG) and calcium are not involved. Using inside-out patches of atrial membrane, the typical response to acetylcholine is mimicked by the application of non-hydrolysable analogues of GTP or the active components of the G-proteins themselves to the internal side of the membrane in the absence of any second messengers. So, either a G-protein exerts a direct effect on the channel or there is a novel intramembrane second messenger system linked to the G-protein (Hescheler et al, 1987).

The inhibition of stimulated calcium influx by U50,488H in rat spinal cord/dorsal

root ganglion co-cultures involves a PT-sensitive G-protein and although this might be mediated indirectly by cAMP under conditions of neurohormonal stimulation, there seems also to be a direct effect of the G-protein on the voltage-dependent calcium channel (Attali et al, 1989).

Rat locus coeruleus neurones are hyperpolarised by morphine by activation of a potassium current, an effect impaired by PT and restored by intracellular GTPγS (Wang and Aghajanian, 1987). AC does not seem to be involved in regulation of potassium channel function in guinea-pig locus coeruleus neurones (Christie et al, 1987; North et al, 1987).

So, Gi/o$\beta\gamma$ (the $\beta\gamma$ subunit dimer of the inhibitory G-proteins Gi and Go respectively) once liberated might act on AC or another second messenger system in the cytosol or could act directly upon a channel protein to cause a conformational change and therefore a change in its activity.

I have found no evidence to implicate adenylate cyclase (AC) as a post-receptor system involved in opiate receptor transduction in isolated SON fragments (see Chapter 4). So, it appears that morphine acts on an opiate receptor linked to a regulatory G-protein, probably Gi or Go, in the cell membrane and that this G-protein does not affect the activity of AC. It is not apparent from the studies in this Thesis which other second messenger systems might be involved in μ-opiate receptor transduction. Nor has this yet been investigated with appropriate intracellular recording/biochemical techniques.

The existence of these many combinations of couplings between the opiate receptors, G-proteins and AC or other second messenger, in a variety of cell types, puts the present findings into context. It seems that how a receptor is linked to a G-protein and then to a second messenger system in the cell depends upon the cell type. The same receptor type might combine differently with these post-receptor elements in different cells. So the present results, namely that the μ- but not the κ-opiate receptor in this system is linked through a PT-sensitive G-protein to intracellular

mechanisms excluding AC, seen on this backcloth are not surprising. What is striking however is the contrast so clearly seen between the two subtypes of opiate receptor present either in the cell membrane of the OT cells themselves or in that of cells projecting to the SON which provide a tonic drive to the OT neurones.

A similar result has been reported in hippocampal and striatal tissue derived from guinea-pigs pre-treated with i.c.v. PT (Werling et al, 1989). The efficacy of opiates acting at the three subtypes of opiate receptor, μ-, δ- and κ- to inhibit noradrenaline release in vitro was assessed. This system has some parallels with that of opiate regulation of SON OT cells as more than one subtype of opiate receptor can mediate an inhibitory response. In guinea-pig hippocampus, PT pre-treatment abolished inhibition of noradrenaline release in response to μ- and δ-agonists whilst not affecting κ-opiate mediated inhibition. So, the μ- and δ-opioid receptors in guinea-pig hippocampus are linked to a PT-sensitive G-protein whereas κ-opioid receptors in the same brain region are unlikely to be so linked. In rat hippocampus, striatum and cortex, again μ-receptor inhibition of catecholamine release was PT-sensitive whereas κ-receptor inhibition was not.

3.4.5. G-proteins and tolerance

The similarity between the effects of PT on sensitivity to morphine and the effects of chronic morphine treatment begs the question, does Gi/o-protein inactivation explain tolerance?

With respect to the results in this Chapter with the κ-opioid receptor agonist, U50,488H, in systems where κ-opiate receptors are shown to be linked to a G-protein, an uncoupling of G-protein from receptor or a reduction in the number of G-proteins available in the membrane might contribute to tolerance to a κ-receptor agonist. Such a mechanism could not explain tolerance to chronic κ-receptor activation in OT cells of the SON (N.B. this has still to be demonstrated) as these receptors are not linked to a PT-sensitive G-protein. This suggests that the phenomenon of tolerance might be a composite of a variety of mechanisms, because tolerance certainly can develop to κ-

opiate receptor agonists in other neurones (Werling et al, 1988). Indeed, we have shown a μ-receptor density down-regulation in the SON after chronic morphine treatment indicating a probable contribution of this mechanism to the observed tolerance (Sumner et al, 1990). Such a change would of course be expected to result in reduced Gi/o-protein activation at a given morphine concentration.

Whatever the cellular mechanisms involved, the consequences of treatment with PT have been likened to the state of tolerance which accompanies chronic administration of opiates (Crain et al, 1986). In cortical membranes from morphine-tolerant guinea-pigs or chronic morphine-treated 7315c pituitary tumour cells, μ-agonist binding is no longer affected by GTP, suggesting a dissociation between the μ-receptor and G-protein (Puttfarcken et al, 1988; Werling et al, 1989). Still more persuasive is the evidence in rat locus coeruleus where chronic morphine treatment results in increased levels of PT-mediated ADP-ribosylation of Gi and Go, which probably represents increased quantities of these inhibitory G-proteins in neurones of the central nervous system (Nestler et al, 1989). Similarly, in guinea-pig myenteric plexus neurones an increase in Gαi/Gαo was demonstrated after chronic fentanyl treatment (Lang and Schulz, 1989).

From the results presented in this Chapter with morphine, coupled with those outlined above, it seems likely that inactivation of the G-protein or a reduction in the number of G-protein molecules available to interact with opiate receptors could be a mechanism underlying tolerance.

Of course, tolerance might be accounted for in isolation from dependence if the two phenomena develop independently, (Wuster and Costa, 1984; Wuster et al, 1985; Christie et al, 1987) by a reduction in G-protein availability. However, in OT neurones of the SON, the existence of tolerance together with dependence has been demonstrated, and they have not been induced independently of one another within the protocol described in Chapter1. With this in mind, the removal of available G-protein (with PT in these experiments) is not sufficient to account for tolerance and

dependence occurring together, as there was no evidence of a withdrawal response to naloxone after PT treatment. The response to naloxone was similar in the control and the PT-treated groups - firing rate was restored to its control level but no further.

So, G-protein inactivation or removal could be one of multiple adaptive mechanisms that together might account for tolerance, but not dependence. Dolphin lists 4 methods by which G-protein modification might result in up or down-regulation of the response to receptor activation. These include a change in the ability of receptor and G-protein to interact; a change in the GTPase activity or affinity for GTP of the G-protein; a change in the effectiveness of the interaction between the α-subunit of the G-protein with the effector molecule and lastly, modification of the number and type of G-proteins available (Dolphin, 1987). Ui and coworkers (Ui et al, 1985) have described the ADP-ribosylation of G-proteins by endogenous ADP-ribosylating enzymes. Thus, Gi is not lost but functionally inactivated by an endogenous mechanism identical to that effected by PT. Further, the regulation of AC in adipocytes is achieved partly by endogenous ADP-ribosylation of Gs (Jacquemin et al, 1986). So PT imitates an existent endogenous homeostatic process which could well contribute to the cellular basis of tolerance after chronic opiate.

In addition to this endogenous ADP-ribosylation process, G-proteins may also be inactivated by phosphorylation by a variety of kinases (Bauer et al, 1987).

So far, I have presented evidence from the literature for association between opioid receptors and G-proteins but have not demonstrated that the G-protein deactivated with i.c.v. PT in this system is linked to a μ-opiate receptor and not to another receptor. It is conceivable that a neurotransmitter receptor present in a neural pathway relaying the effects of an acute i.v. injection of morphine to the SON OT cells is the target for PT or more broadly, that heterologous desensitisation at several receptor sites other than just the μ-opiate receptor occurs as the pool of available G-proteins is depleted after PT.

Crain and coworkers using mouse spinal cord-dorsal root ganglion cultures (Crain

et al, 1986) found that agonists at receptors other than opiate receptors which are also linked to a PT-sensitive G-protein are less potent after PT-treatment indicating a common pool of G-protein able to interact with a variety of receptors. This situation cannot be ruled out in SON OT neurones but neither can it be supported with experimental evidence from these studies. Although this possibility cannot be discounted, because the potency of agonists at other putative neurotransmitter receptor sites was not systematically tested, before and after PT treatment, the firing rate of OT cells in vitro in the hypothalamic slice preparation is potently inhibited by opiates acting at the μ-opiate receptor (Pittman et al, 1980; Wakerley et al, 1983; Pumford et al, 1987) and that μ- and κ-opiate receptors are located in the SON, although it is not yet clear which neural elements carry them (Sumner et al, 1990). So it is probable that a G-protein associated with supraoptic μ-opiate receptors is deactivated by i.c.v. PT treatment, perhaps sufficiently to account for the observations described in this Chapter, with the implications discussed above.

Although opiate tolerance has been demonstrated in cultured neurones indicating that the necessary elements that contribute to the development of tolerance can be present in an individual cell (Sharma et al, 1975a; Sharma et al, 1975b), tolerance might also be the result of adaptive changes at distinct sites in the brain which influence each other to produce the gross evidence of tolerance in terms of cell function. The influence of the AV3V region upon the expression of withdrawal in the chronic morphine-treated rat has been explored in this Thesis (see Chapter 6). The tolerance of AV3V-lesioned rats to morphine could not be tested as was done in this series of experiments, because the lesion itself removes a tonic excitatory input and silences the SON OT neurones. This could be investigated however in AV3V-lesioned rats given i.v. cholecystokinin to raise the basal electrical activity to a level upon which inhibitory effects might be seen, using a similar protocol to that used here, (Blackburn and Leng, 1990).

If a range of doses of i.c.v. PT had been tested, to assess the maximum extent of

PT-induced 'tolerance' attainable, it might have been possible to then predict the likely maximal contribution of this potential adaptive mechanism to chronic opiate-induced tolerance. However, at the dose used some morphine-induced inhibition of spontaneous activity was seen, albeit attenuated. It seems possible therefore that receptor down-regulation (see Chapter 5) contributes significantly to tolerance.

3.4.5. A possible mechanism for morphine-induced low dose excitation

As previously demonstrated in Chapters 1 and 2, excitatory and inhibitory effects of morphine were seen in PT-treated rats. In the control i.c.v. 0.9% saline-treated group excitatory effects were seen between 1 and 11μg/kg and in the i.c.v. PT-treated group between 1 and 661μg/kg. The threshold dose to inhibition was between 11 and 61μg/kg in the i.c.v. 0.9% saline-treated group and between 1 and 1661μg/kg in the i.c.v. PT-treated group. The latter comparison is not surprising given the shift in responsiveness to acute i.v. morphine already described earlier and illustrated in Figure 3.4.1.. The former comparison is difficult to interpret, unless the excitatory effects of morphine are not mediated by a PT-sensitive G-protein. In 2 PT-treated rats, excitatory effects were seen up to 661μg/kg morphine. In the remainder excitatory effects were seen between 1 and 61μg/kg morphine which is not dissimilar to the range observed in the control group and previously seen in Chapters 1 and 2. Note that the lower end of this range is 1μg/kg morphine, so cells can still be responsive at this very low dose of morphine after PT treatment. The spread of effective excitatory doses of morphine in the PT-treated group however raises the question, how does the deactivation of Gi/o affect the expression of the excitatory effects of morphine? One interpretation of the results is that with the inhibitory potency of morphine impaired after PT, the inhibition which always follows low dose excitation with morphine is not activated until sufficient of the now scarce G-protein becomes recruited in the usual way and this now occurs at the higher doses of morphine. Normally the inhibitory effect of a high dose of morphine will overcome the sometimes persistent excitatory effect of a lower dose. The low dose excitatory

effects of morphine do not seem to involve a PT-sensitive G-protein: this would explain the effectiveness of 1μg/kg morphine in exciting 3 cells from the PT-treated group. Perhaps a dual mechanism has been dissected by which morphine can act on these neurones: an excitatory effect not involving a PT-sensitive G-protein and an inhibitory effect involving Gi/o occurring at higher concentrations of morphine at the receptors. This dual effect could be the result of activation of two distinct subtypes of μ-opioid receptor to which morphine binds with differing affinity one of which associates with a PT-sensitive G-protein and the other not and perhaps one of which is located presynaptically and one postsynaptically. So with the receptor mediating inhibition by morphine rendered 'tolerant' after PT, the excitatory effects mediated usually just at low doses of morphine by the other receptor can be evoked at higher doses because of the removal of the inhibitory restraint usually present as the dose of morphine increases. PT treatment reduces the availability of Gi/oβ (the β subumit of the inhibitory G-proteins Gi and Go respectively; Watkins et al, 1989) and if a stimulatory G-protein Gs was associated with the other subtype of μ-opioid receptor proposed in this model, then as Gi/oβ subunit levels decline, the excitatory effects mediated by Gs might be unmasked.

This scenario is highly speculative in this instance because the number of cells involved is small and the experiments were not designed to investigate this phenomenon primarily. There is as yet no evidence for the existence of subtypes of the μ-opioid receptor on these cells, although they have been postulated in other cells (Lutz et al 1985; Pasternak and wood 1986; Rothman et al 1987). But this intriguing result merits more study.

Dual effects of opiates at different dose ranges have already been demonstrated in neurones and have been explained in terms of association of receptors with different G-proteins, Gs (a stimulatory G-protein) and Gi/o (Crain and Shen, 1990). Indeed it is possible that when an inhibitory receptor is activated an interaction occurs between the released $\beta\gamma$-subunit complex of Gi/o and free Gsα (the α subunit of the stimulatory G-

protein, Gs) , to reduce the availability of the excitatory G-protein Gs, thus indirectly producing inhibition (Katada et al, 1984; Nestler et al 1989). Such a hypothesis was proposed by Nestler and coworkers based on experimental results in morphine-tolerant rats, which showed an increase in the levels of Giα/Goα with no change in the levels of the β-subunits of Gi/Go. However, these results conflict partially with those of Lang and Schulz (1989) who demonstrated not only an increase in the levels of Giα/Goα but also an even more pronounced increase in Giβ/Goβ and a decrease in the level of Gs in guinea-pig myenteric plexus.

More problematic is the reconciliation of the effect of chronic morphine treatment upon inhibitory G-protein levels and the effect of the same treatment upon AC activation. The increase in Gi/o in the tolerant state would be expected to cause attenuation of the activation of AC. In fact AC activation in both systems described above is increased after chronic morphine treatment to 'normal' levels. Lang and Schulz explain this paradox by implying that AC is not directly involved in the cell response to chronic opiate whereas regulation of inhibitory G-protein is, and therefore the change in AC observed after chronic opiate is coincidental and not a direct effect.

Nestler and coworkers on the other hand try to encompass both observations by proposing a model by which the two systems might be so affected. If both the stimulatory and inhibitory G-protein subunits αs and αi share the same pool of $\beta\gamma$, then under basal conditions, an equilibrium is established resulting in basal concentrations of free αs and αi with their resultant influence on AC. Acute morphine would decrease the probability of association between $\beta\gamma$ and αi and increase the probability of association between $\beta\gamma$ and αs thus freeing relatively more αi which would depress the activity of AC. After chronic morphine treatment, assuming that $\beta\gamma$ is unchanged (not the case in myenteric plexus, Lang and Schulz, 1989) then, as the quantity of αi increases, but that of αs remains unchanged, $\beta\gamma$ would associate mainly with αi according to the equilibrium, thus freeing relatively more αs to activate AC, resulting in the observed increase in activity of AC. Again, this scheme relies

upon the quantity of αs remaining unchanged throughout, which is a point of controversy (see Lang and Schulz, 1989). The scheme applies to Go as well and gives rise to the idea that Gs can be regulated indirectly by controlling the levels of Gi/Go.

U50,488H was not tested over the same dose range as morphine; usually only the second half of the dose range was tested and this only in the PT-treated group. For this reason there is not the same information for U50,488H as exists for morphine on low dose excitation, a feature of the effect of U50,488H on supraoptic OT cells (see Chapter 2).

The fact that both morphine and U50,488H acted in a fully naloxone-reversible way at usual doses of naloxone indicates that the active sites on the opiate receptors were not in any way modified by PT treatment and this could not account for the observed shift in sensitivity of OT cells to i.v. morphine.

Whether PT-treatment would have an effect upon the development of tolerance to morphine (not addressed by the present study) might be answered using a similar protocol to that used in Chapter 1, but where PT was administered simultaneously with the start of a chronic i.c.v. infusion of morphine. This might illuminate more fully the involvement of G-proteins in acute and chronic effects of opiates upon the OT neurosecretory system.

CHAPTER 4

INTRACELLULAR cAMP AND OXYTOCIN AND VASOPRESSIN RELEASE WITHIN THE RAT SUPRAOPTIC NUCLEUS (IN VITRO): ARE THEY AFFECTED BY ACUTE (IN VITRO) OR CHRONIC (IN VIVO) MORPHINE TREATMENT?

4.1. INTRODUCTION

The cellular events underlying the acute and chronic effects of opiates have been the subject of much investigation. Intracellular calcium has been implicated in the response of cells to acute and chronic opiate exposure but the relationship is still not clearly defined. Opiates are known to inhibit neurotransmitter release in various systems and as neurotransmitter release is known to be a calcium-dependent process (for review see Llinas and Heuser, 1977) the possibility that opiates inhibit the depolarisation induced influx of calcium into nerve terminals, thus reducing neurotransmitter release has received much support. Alternatively, the inhibition of calcium influx by opiates could lead to membrane hyperpolarisation and relative insensitivity of the neurone to depolarising stimuli (West and Miller, 1983).

cAMP generation has also been frequently investigated in the presence of acute and chronic opiates and there is a strong case at least in some cells, for a role of adenylate cyclase (AC) in mediating the effects of opiates on the cell. In the supraoptic nucleus (SON) in vitro the opioids, met- and leu-enkephalin and β-endorphin inhibit the formation of cAMP (Redgate et al, 1986). Agonists at the κ-opiate receptor do not stimulate cAMP formation in guinea pig striatal membrane (De Montis et al, 1986). δ-opiate receptors do not occur in the SON (Mansour et al, 1987; Tempel and Zukin, 1987; Mansour et al, 1988; Sumner et al, 1990) and because of the extremely low preference of the ligands met- and leu-enkephalin and β-endorphin for the κ-receptor (Kosterlitz, 1985), it appears that the inhibition of cAMP generation was probably mediated through the μ-type opiate receptor. Unfortunately, a study using more selective ligands has not been done to date in the SON. However, in the 7315c pituitary tumour cell line, which has a population of exclusively μ-type opiate receptors, the μ-selective agonist, Tyr-D-Ala-Gly-MePhe-Gly-ol (DAGO) inhibits AC acutely (Puttfarcken et al, 1988). So activation of μ-type opiate receptors can lead to inhibition of AC although this might not extend to all cell types.

As a consequence of opiate induced changes in Ca^{2+} concentration or cAMP formation , protein phosphorylation by enzymes activated by cAMP or by Ca^{2+} might also be part of the cellular compensatory mechanisms that constitute tolerance and dependence (West and Miller, 1983).

The acute and the chronic effects of opiates in neuronal and in cultured neuroblastoma x glioma cells have been linked to changes in intracellular cAMP. Acutely cAMP production is inhibited by opiates but with continued exposure cAMP concentration returns to basal as a result of hyperactivity of AC (Klee et al, 1975). The possibility of explaining the acute effects of opiates in terms of changes in cAMP formation and that such adaptive changes in the AC system might occur in the neurosecretory neurones of the SON to explain the powerful chronic effects of opiates there, has not so far been investigated. In this study, the relatively μ-selective opiate receptor ligand, morphine was used, which has negligible affinity for the κ- or the δ-type opiate receptor (Kosterlitz, 1985), in an in vitro tissue incubation protocol involving the measurement of the tissue content of cAMP and the release into the superfusate of cAMP, oxytocin (OT) and vasopressin (AVP) to answer the following questions:

1. Does morphine affect cAMP concentration or release in SON; that is, are μ-opioid receptors negatively coupled to AC in this neurosecretory system as they are elsewhere and is this effect naloxone-reversible?

2. Do the SON from morphine-treated rats have a different cAMP content to SON derived from morphine-naive rats?

3. Do endogenous opioid peptides maintain an inhibitory tone on intracellular cAMP concentration or on AVP or OT release within the SON?

4. Are SON prepared by this method and treated according to this protocol capable of responding to stimulation of AC by an agent (VIP) proven stimulatory in an earlier study in SON? This would confirm that VIP is positively coupled to AC and that the tissue is responding as expected.

5. Can morphine antagonise the expected stimulation of cAMP by VIP? Thus the effect of morphine superfusion on both basal and stimulated cAMP formation was tested.

6. Are there any changes in neuropeptide release from SON in vitro with acute opiate exposure or in SON derived from rats chronically i.c.v. morphine-infused, after naloxone in vitro? In vivo, there is a sharp and dramatic rise in plasma OT concentration associated with precipitated withdrawal in chronic morphine-treated rats. AVP release however is largely unaffected (Bicknell et al, 1987b; Bicknell et al, 1988a). With the present protocol, the question was addressed of whether OT and AVP release from dendrites or other neural processes within the SON also occurs in conditions designed to be similar to those described above, which provoke a substantial release of OT into the systemic circulation.

4.2. METHODS

The study comprised two series of experiments which focussed on two aspects of opiate action in the SON. First, acute affects of morphine, naloxone and VIP were examined in SON derived from morphine-naive control rats (Experiment 1). cAMP formation and OT and AVP release into the superfusate were measured.

Second, acute and chronic effects of morphine upon cAMP formation and OT and AVP release within the nucleus were studied in SON derived from either morphine-naive rats or from 5-day i.c.v. morphine-infused rats and finally the acute effects of naloxone on tissue cAMP and release of OT and AVP in tissue taken from 5-day i.c.v. morphine-infused rats (Experiment 2a and 2b respectively) were studied. The two experimental protocols differed slightly and are dealt with separately in both the Methods section and in the Results section.

4.2.1. Experiment 1

4.2.1.1. Animals

Rats were virgin female Sprague Dawley, housed at an ambient temperature of 21-23°C, allowed free access to food (standard breeder diet) and tap water and kept under a 13/11h light/dark cycle. The mean ± SEM body weight of these rats was 228.7 ± 2.4g (n = 24).

4.2.1.2. Preparation of tissue

Rats were decapitated by guillotine. The skull vault was opened, the dura incised in the midline and a transverse cut was made rostrally to the optic chiasm. The optic nerves were cut with minimal stretching and a caudal cut made at the cerebellum, to free the block containing the hypothalamus. The brain was drenched with ice cold modified Yamamoto's solution and further trimmed at the optic nerves and the mammillary bodies. This block of tissue was fixed with cyanoacrylate adhesive to the chuck of the Vibraslice rostral end uppermost and immediately immersed in Yamamoto's solution in the Vibraslice chamber. Coronal 500μm sections were cut

and the three sections containing SON were transferred to a plastic Petri dish containing Yamamoto's solution and placed on ice. With the aid of a dissecting microscope and using a fragment of razor blade held in a pin vice the area containing the SON in each section was isolated. The isolated sections of SON from the left and right hand sides of the brain were placed into separate tared inverted plastic tube caps filled with Yamamoto's solution. Weighing was carried out once the cap was attached to the tube and the weight of tissue calculated by later reweighing of the tube and cap on a microbalance. The tissue was then transferred to the superfusion chamber which was inserted into the primed superfusion apparatus and carefully sealed, excluding air bubbles. The contralateral SON were paired as control and experimental tissues.

4.2.1.3. Superfusion apparatus

This comprised a series of chambers made of perspex and modelled from a 2ml disposable syringe. Each chamber was 150μl capacity. The tissue was supported on a fine nylon mesh. The outlet of the chamber was connected via a needle connector and polythene tubing through a holder so that after passing over the tissue the superfusate dripped into tared plastic tubes. When sealed by careful insertion of a plunger with a rubber O ring to make a good seal (see Figure 4.2.1.4.), each chamber was connected via the plunger inlet independently and via a bubble trap to a line of an 8 channel peristaltic pump (Gilson). The inlet end of each line was immersed into the appropriate superfusion medium. Each such reservoir was gassed with 95%O_2/5%CO_2. The superfusion chambers and inlet lines were kept at 37°C in a stirred water-jacket heated via an immersion heater, thermostatically controlled. The bath temperature was set so that the superfusion chamber temperature, measured with a thermocouple was 37°C.

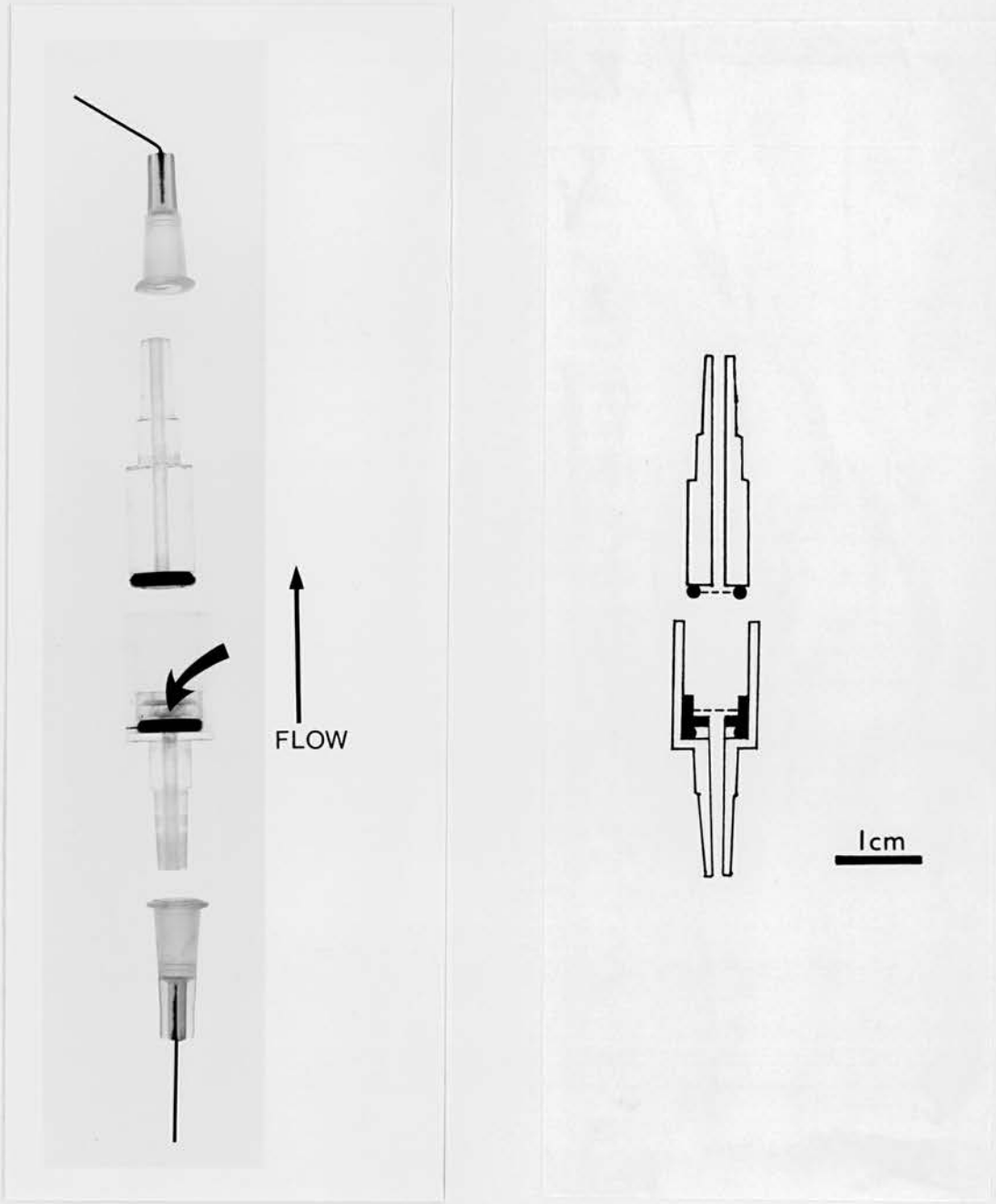


Figure 4.2.1.4. Superfusion apparatus

See Methods Section 4.2.1.3. for full description. The curved arrow indicates the inside of the 150μl chamber bounded at its lower surface by a nylon mesh stage resting on a rubber O-ring. The fragments of SON were placed on this surface and then the plunger inserted to seal the tissue in. The upper surface of the chamber was also constructed from fine nylon mesh attached to the upper O-ring. The medium flowed from the lower surface, through the chamber and out through the upper surface, as depicted by the vertical arrow. The lower and upper components terminated in a male Luer fitting and needles, which were themselves connected to inlet and outlet tubing, were attached.

4.2.1.5. Superfusion protocol

The superfusate flow rate was set for each channel at 50μl/min, calibrated gravimetrically. The transit time of this system was estimated at 4min.

All tissues equilibrated for 90min in the chamber during which time they were superfused either with Yamamoto's medium containing the peptidase inhibitor, bacitracin (2.8mg/100ml; Sigma Chemical Co.; first control group) or with Yamamoto's medium containing bacitracin together with the phosphodiesterase inhibitor, IBMX (isobutyl methylxanthine, 1mM; Sigma Chemical Co.; all other treatment groups). The first control group was not exposed to IBMX in order to show the degree of effectiveness of the phosphodiesterase inhibitor and to enable comparison between Experiment 1 and Experiment 2. During the equilibration period, no drugs were present in the superfusate. Subsequently, 5 - 6 serial collections of superfusate were made from each chamber, each over 10min, into tared 1.5ml Eppendorf tubes. After the 30min control period during which superfusate samples were collected for radioimmunoassay for OT and AVP drugs were added to the superfusate for the duration of the remainder of the protocol and three 10min collections of superfusate made. A final collection of superfusate was made after removal of the tissue from the chamber to provide medium blanks to check for cross reactivity of drugs in the radioimmunoassay.

Drugs were morphine sulphate B.P. (10^{-5}M; Pharmacy, Edinburgh Royal Infirmary), naloxone hydrochloride (10^{-6}M; a gift from Endo Labs. or purchased from Sigma Chemical Co.) and vasoactive intestinal peptide (VIP; 10^{-6}M; human/porcine/rat (His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH_2), Cat. code 7161, Peninsula Labs. Inc.), a peptide known to elevate cAMP in SON and other tissues (Redgate et al, 1986).

4.2.1.6. Treatment of superfusates

The full volume of superfusate collected over 10min was frozen at -20°C for later radioimmunoassay of OT and AVP. None was reserved for radioimmunoassay of cAMP in view of the negative results from the parallel experiments (Experiment 2).

4.2.1.7. Treatment of tissues

At the end of the last 10min collection period, the chamber was opened, the fragments of SON were removed (and the number of fragments checked) and placed into 500μl ice cold 70% ethanol in a chilled 1ml glass minihomogeniser, dispersed, left for 20min in the minihomogeniser on crushed ice, then tranferred to a 1.5ml Eppendorf tube and centrifuged for 10min at -20°C and 6000r.p.m. (Heraeus-Christ). The supernatants were transferred to glass flat bottomed tubes and dried in a stream of nitrogen at 40°C in a dry heating block. Residues were re-dissolved in 500μl 10mM phosphate buffered saline (PBS), pH 7.4, then divided into two aliquots, frozen and stored at -20°C prior to assay of cAMP content. Tissue blanks were prepared containing either PBS alone or 2μl of each of the drug solutions used (equivalent to the volume of SON) and processed as for tissue; these were used to test for cross-reactivity in the radioimmunoassay.

4.2.2. Radioimmunoassay for oxytocin and vasopressin

4.2.2.1 Oxytocin (OT)

The OT assay was expertly carried out by Chris Chapman, Dept. Neuroendocrinology, AFRC Institute of Animal Physiology and Genetics Research, Babraham, Cambridge.

a) Stock buffer:

A stock RIA buffer containing sodium phosphate 0.01M (pH 7.4), bovine serum albumin 5mg/ml (Sigma Chemical Co.), sodium azide 1mg/ml and bovine gamma globulin 1mg/ml (G5009, Sigma Chemical Co.) was used for dilutions where indicated.

b) Assay procedure:

Samples were prepared as described above and slowly thawed from -20°C at 4°C, vortexed and recentrifuged. Aliquots of 50μl were taken for radioimmunoassay. All reagents were diluted in stock buffer apart from 30% PEG 6,000 which was diluted in water. The RIA standard curve was constructed using synthetic oxytocin (PB2232A, Cambridge Research Biochemicals, U.K.) which was dissolved in 0.2% acetic acid to a final concentration of 1mg/ml. This was then stored in 25μl aliquots at -20°C.

The standard curve was contructed by making serial doubling dilutions of synthetic oxytocin in stock assay buffer over the range 25,000 - 6 pg/ml. 50μl aliquots of standard and 50μl of sample were added to assay tubes in duplicate. 50μl oxytocin antiserum (Higuchi et al, 1985) diluted in stock assay buffer to a final dilution of 1:300,000 was added to each assay tube which was then vortexed and incubated at 4°C for 24 hours. ^{125}Iodinated oxytocin was diluted in stock assay buffer to obtain 8,000 c.p.m./50μl and 50μl aliquots of this solution were added to each assay tube, the tubes vortexed and incubated at 4°C for 48 hours.

At this stage, 150μl 30% PEG 6,000 was added to each tube and the tubes vortexed and centrifuged for 30min at 3000 r.p.m. and 4°C. The supernatant was aspirated off and the radioactivity of the remaining pellet counted in a gamma counter (Packard 5360 Auto-Gamma).

4.2.2.2 Vasopressin (AVP)

The AVP assay was expertly carried out by Chris Chapman, Dept. Neuroendocrinology, AFRC Institute of Animal Physiology and Genetics Research, Babraham, Cambridge.

a) Stock assay buffer:

A stock RIA buffer containing sodium phosphate 0.05M (pH 7.4), sodium chloride 0.1M, sodium azide 1mg/ml, bovine serum albumin 1mg/ml (Sigma Chemical Co.) and bovine gamma globulin 1mg/ml was used for dilutions where indicated.

b) Assay procedure:

Superfusates, frozen upon collection were thawed slowly at 4°C prior to assay and any precipitate removed by centrifugation. AVP was extracted (Sep-pak C18 cartridge; Waters Associates, Millipore, Harrow, Middlesex) and the sample dried down under vacuum (Gyrovap sample concentrator; V. A. Howe Distributors) to remove excess methanol and acetic acid used in the extraction procedure. AVP samples once extracted were reconstituted in 150μl stock assay buffer and 50μl aliquots of this used for RIA. Synthetic AVP (PB2279A; Cambridge Research Biochemicals, U.K.) dissolved in 0.2% acetic acid to a final concentration of 1mg/ml was used to contruct the standard curve. 10μl aliquots of this were stored at -20°C.

The standard curve was constructed from doubling dilutions of synthetic AVP in stock assay buffer over a concentration range of 4,000 - 2pg/ml. 50μl aliquots of sample and 50μl aliquots of standard were added to assay tubes in duplicate. 50μl AVP antiserum (Moore et al, 1977) diluted in stock assay buffer 1:30,000 was added to each tube and tubes were vortexed and incubated for 24 hours at 4°C. ^{125}Iodinated AVP was diluted in stock assay buffer to obtain 8,000c.p.m. /50μl and 50μl aliquots of this were added to each assay tube. Tubes were vortexed and then incubated for 48 hours at 4°C. 150μl 30% PEG 6,000 was added to each tube, tubes vortexed and then centrifuged for 30 min at 3,000 r.p.m. and 4°C. The supernatant was aspirated and the remaining radioactivity counted in a gamma counter.

4.2.2.3 Calculation of results

The standard curve was used to measure the amount of hormone in each sample. Also making up the standard curves were tubes containing no antiserum (as a measure of non-specific binding), total counts tubes and total binding tubes (containing no competing unlabelled hormone). The standard curve was linearised by computer using the logit transformation to give a non-weighted linear equation:

$\text{logit}X = \log_e X(1-X)^{-1}$

where X is the proportion of bound label. Unknown hormone concentrations were

calculated from the calibration curve using the mean count from two replicate tubes for each sample.

4.2.2.4. Assay coefficients of variation

Standard hormone solutions were assayed at 200pg/ml in the appropriate assay buffer. These standards were assayed as 8 sets of duplicates and the hormone concentrations measured used to calculate the intra- and inter-assay coefficients of variation. The intra- and inter-assay coefficients of variation (the sample standard deviation expressed as a percentage of the sample mean) were calculated from:

$$\sigma/\mu \times 100,$$

where σ=sample standard deviation and μ=sample mean of all intra- or inter-assay standards.

For the oxytocin RIA the intra-assay coefficients of variation were 10.4% and 14.1% and the inter-assay coefficient of variation 9.0%. For AVP the intra-assay coefficients of variation were 11.6% and 13.2% and the inter-assay coefficient of variation7.6%.

4.2.2.5. Sensitivity of the radioimmunoassays

The sensitivity for each assay was determined from 2 x the standard deviation about the mean of the zero binding point. For oxytocin this was 0.2-0.4 pg/tube. For AVP sensitivity was 0.4 pg/tube.

4.2.3. Radioimmunoassay for cAMP

The assay was expertly performed by Martin Hulme, Reproductive Biology Unit, Chalmers Street, Edinburgh.

Samples were thawed slowly at 4°C and vortexed. 100μl aliquots were taken for radioimmunoassay.

a) Assay buffer:

Phosphate buffered saline (PBS; 10mM; pH 7.4) was used where indicated to dilute samples and standards.

In order to make the assay more sensitive, all tubes, including non specific binding

(NSBs) and maximum binding (Bos) were acetylated, to make the cold cAMP resemble the succinylated cAMP-^{125}I-tyrosine methyl ester label (Cailla et al, 1973). Acetylation was performed using 2.7ml triethylamine and 1.0ml acetic anhydride made freshly prior to use of which 5μl was added to each tube. Tubes were vortexed and left to stand for at least 1 hour.

b) Standard curve:

The standard curve was constructed using anhydrous cAMP made up in a range of concentrations in ethanol, evaporated under nitrogen and reconstituted in 200μl assay buffer. The range was 10fmol to 2500fmol cAMP/tube.

c) Assay procedure:

100μl antiserum raised against a conjugate of human serum albumin and succinyl cAMP (MS/1 or MS/3; Steiner et al, 1972), diluted 1:20,000 in 0.3% bovine gamma globulin in PBS was added to all tubes in duplicate except total counts (TCs) and NSBs. NSBs received 100μl 0.3% bovine gamma globulin in PBS instead of antiserum.

All tubes then received 100μl cAMP label, c 30,000cpm in PBS (iodination was by the chloramine T method). TCs were covered with parafilm. All tubes were vortexed and incubated overnight at 4°C. Free and bound label were separated using 1.6ml 16% polyethylene glycol 6000 (made up in distilled water). Tubes were centrifuged at 2000rpm for 30min at 4°C and the supernatant discarded. Radioactivity of the remaining pellets was counted using a Nuclear Enterprises 1600 multihead gamma counter and results calculated using log-logit RIA analysis.

4.2.4. Experiment 2

4.2.4.1. Animals

Experiment 2a

Virgin female untreated Sprague Dawley rats were used from the same batch and immediately before those in 2b.. The mean ± SEM body weight of these rats was 242.7 ± 4.6g (n=10).

Experiment 2b

Virgin female Sprague Dawley rats were implanted under ether anaesthesia with an intracerebroventricular cannula, connected via polythene tubing to a subcutaneous osmotic minipump to deliver morphine as described in detail in Chapter 1, Methods section. These rats were weighed daily to check their well-being and rectal temperature was recorded daily to check that the morphine infusion system was operating since body temperature is increased by i.c.v. morphine infusion. Mean body weight (± SEM) of these rats was 253.7 ± 7.4g (n=6) on the day of implantation of the i.c.v. cannula and 258.3 ± 6.8g (n=6) on the day they were killed for collection of SON.

4.2.4.2. Preparation of tissue

Tissue was collected and prepared for incubation as described in 4.2.1.2. in all respects apart from tissue taken from 5-day i.c.v. morphine-infused rats which was sectioned in Yamamoto's solution containing morphine (10^{-5}M).

4.2.4.3. Superfusion apparatus

The apparatus used for this series of experiments was identical to that described in 4.2.1.3. and is shown in Figure 4.2.1.4..

4.2.4.4. Superfusion protocol

All tissues were left for 90min to equilibrate in the chamber during which time they were superfused with Yamamoto's medium containing bacitracin (2.8mg/100ml; Sigma Chemical Co.), but without IBMX (tissue derived from morphine-naive rats) or with Yamamoto's solution, without IBMX but with added morphine, 10^{-5}M (tissue derived from 5-day i.c.v. morphine-infused group). Superfusate was collected at 10min intervals into tared 1.5ml Eppendorf tubes. Drugs to be acutely added were included after the 30min control superfusate collection period and two superfusate samples collected during superfusion with drug-containing medium.

2a) Acute and chronic effects of morphine on cAMP formation and OT/AVP release

The chamber containing fragments of one SON (left or right, randomnly selected) from each rat was superfused with medium as detailed above, containing morphine (10^{-5}M) throughout when tissue was derived from a chronic i.c.v. morphine-infused rat. Three groups made up this series of experiments and they are listed below together with the question they were designed to address:

i. CONTROL GROUP I:

'What are the basal levels of cAMP formation and OT/AVP release in SON?'

tissue from morphine-naive rats

no drugs in vitro

ii. CONTROL GROUP II:

'What are the acute effects of morphine on these basal levels of hormone and second messenger production in normal SON?'

tissue from morphine-naive rats

morphine (10^{-4}M) in vitro for test 30min

iii. I.C.V. MORPHINE-INFUSED 'CONTROL' GROUP I:

'What are the effects of chronic morphine on cAMP formation and OT/AVP release in SON?'

tissue from 5-day i.c.v. morphine-infused rats

morphine (10^{-5}M) throughout in vitro

Superfusate was changed after the first three collection periods (i.e. 30min) to Yamamoto's medium containing morphine 10^{-4}M (intended to maximize any acute effect of morphine) for control group II.

2b) Effect of acute naloxone on cAMP formation and OT/AVP release in SON taken from morphine-naive and chronic i.c.v. morphine-infused rats.

For tissue from these rats, the superfusate always contained 10^{-5}M morphine; (this concentration is similar to that found in the hypothalamus in vivo following i.c.v. infusion of morphine for 5 days (Rayner et al, 1988)), but no IBMX, as described above. The chamber containing fragments of SON (left or right, randomly selected) from each rat was superfused with this medium until the control 30min had passed, when for test chambers, the superfusate was changed to medium containing naloxone 10^{-5}M as well as 10^{-5}M morphine and two further superfusate samples collected.

So, two groups made up this series of experiments and they are listed below together with the question they were designed to address:

iii. I.C.V. MORPHINE-INFUSED 'CONTROL' GROUP I:

'What are the effects of chronic morphine on cAMP formation and OT/AVP release in SON?'

tissue from chronic i.c.v. morphine-infused rats

morphine (10^{-5}M) throughout in vitro

iv. I.C.V. MORPHINE-INFUSED / ACUTE NALOXONE-TREATED GROUP

'What are the acute effects of naloxone on cAMP formation and OT/AVP release in SON tolerant to morphine after chronic i.c.v. infusion?'

tissue from 5-day i.c.v. morphine-infused rats

morphine (10^{-5}M) throughout in vitro

naloxone (10^{-5}M) in vitro for test 30min

Group iii. serves as a test group for experiment 2a and a control group for experiment 2b and there were 4 groups in total studied in experiment 2. Tissue blank channels were included in which chambers were superfused with test media in the absence of SON to check for cross reactivity of drugs in the radioimmunoassays.

4.2.4.5. Treatment of superfusates

Superfusates were treated as described in 4.2.1.6. and some superfusate was retained for cAMP radioimmunoassay.

4.2.4.6. Treatment of tissues

Tissues were treated exactly as described in 4.2.1.7. and tissue content of cAMP was measured by specific radioimmunoassay.

4.2.5. Radioimmunoassay for OT and AVP

Radioimmunoassay of OT and AVP was exactly as described in 4.2.2..

4.3. RESULTS

4.3.1. Experiment 1: cAMP formation in SON taken from morphine-naive rats: effects of superfusion with morphine, naloxone and VIP.

cAMP immunoreactivity was detectable in tissue fragments from all treatment groups. The inclusion of the phosphodiesterase inhibitor IBMX in the superfusate of the second control group and all subsequent treatment groups, increased cAMP content measured under control conditions. Total cAMP was increased significantly (P=0.03, Wilcoxon test for independent samples) upon inclusion of IBMX in the superfusate but cAMP concentration was not significantly different (Wilcoxon test for independent samples) to that in the first control group (without IBMX). Total cAMP increased to 169.8% from 416.1 ± 124.8 to 706.4 ± 98.0 fmol/SON and concentration of cAMP to 130.9% of the value in the absence of IBMX from 464.1 ± 216.3 to 607.5 ± 131.6 fmol/mg tissue (not significantly different, Wilcoxon test for independent samples).

Addition of morphine (10^{-5}M) to the superfusate had no effect on cAMP formation in the tissue. Total content was 792.4 ± 98.0 fmol and concentration 784.7 ± 200.4 fmol/mg neither of which was significantly higher than control values (P=0.56 for both comparisons, Wilcoxon test for independent samples). This result agrees with that found in experiment 2 where IBMX was not included in the superfusate: acute exposure to morphine does not have any effect on formation of cAMP in the SON under these conditions.

Superfusion with naloxone alone (10^{-6}M) resulted in a slight rise in the content and concentration of cAMP to 1025.0 ± 113.3 fmol and 847.6 ± 101.7 fmol/mg SON but in neither case was the increase significantly beyond that of the control group: naloxone alone had no effect on the formation of cAMP in morphine-naive tissue.

Predictably, the addition of morphine and naloxone simultaneously to the superfusing medium had no effect on cAMP production.

In contrast to the negative results thus far obtained, VIP caused a massive increase in cAMP formation in terms of content and concentration. Total cAMP was 4849 ± 954.8 fmol and the concentration of cAMP was 3942 ± 816.1 fmol/mg SON, increases to 686.3% and 649.0% control values. These two measures differed considerably from control ($P = 0.008$ and $P = 0.012$ respectively, Wilcoxon test for independent samples). The stimulation of cAMP production was not attenuated by the concurrent superfusion of morphine (10^{-5}M) and this treatment also resulted in cAMP formation at a level greater than control at 5727 ± 1261 fmol and 5999 ± 1471 fmol/mg respectively, 810.8 and 987.6% control values. This was different from control in both cases ($P = 0.002$ and $P = 0.002$ respectively, Wilcoxon test for independent samples) but not from that of VIP alone.

Table 4.3.1.1. Experiment 1: cAMP-like immunoreactivity in SON taken from morphine-naive rats; effects of superfusion with morphine (10^{-5}M), naloxone (10^{-6}M) and VIP (10^{-6}M).

Treatment group	Tissue weight (mean±SEM; mg)	Total cAMP (mean±SEM; fmol)	cAMP/mg tissue (mean±SEM; fmol/mg)
a) control (n=6) No drugs (+)bacitracin (-)IBMX	0.97 ± 0.14	416.07 ± 124.76	464.10 ± 216.29
b) control (n=8) No drugs (+)bacitracin (+)IBMX	0.86 ± 0.11	706.39 ± 97.96	607.48 ± 131.63
c) (n=6) (+)morphine 10^{-5}M (+)bacitracin (+)IBMX	0.84 ± 0.12	792.44 ± 106.79	784.68 ± 200.37
d) (n=6) (+)naloxone 10^{-6}M (+)bacitracin (+)IBMX	1.00 ± 0.17	1024.97 ± 113.26	847.64 ± 101.68
e) (n=6) (+)morphine 10^{-5}M (+)naloxone 10^{-6}M (+)bacitracin (+)IBMX	0.87 ± 0.15	815.91 ± 128.42	778.24 ± 239.09
f) (n=6) (+)VIP 10^{-6}M (+)bacitracin (+)IBMX	0.80 ± 0.08	4848.96 ± 954.81	3942.29 ± 816.13
g) (n=6) (+)VIP 10^{-6}M (+)morphine 10^{-5}M (+)bacitracin (+)IBMX	1.00 ± 0.11	5727.22 ± 1260.69	5999.49 ± 1471.36

Table 4.3.1.1. cAMP content and concentration are expressed as mean values ±

standard error of the mean, group size, n. There was no difference from control in the groups exposed to opiate and/or opiate antagonist (Kruskal-Wallis) but the tissues superfused with VIP (10^{-6}M) responded with a dramatic increase in tissue cAMP content and concentration (P=0.008 and P=0.012 respectively, Wilcoxon test for independent smples). Likewise, those tissues superfused with VIP (10^{-6}M) and morphine (10^{-5}M) simultaneously also responded with a clear increase in cAMP content and concentration (P=0.002 and P=0.002 respectively, Wilcoxon test for independent samples). All tissues received bacitracin. IBMX was included where stated.

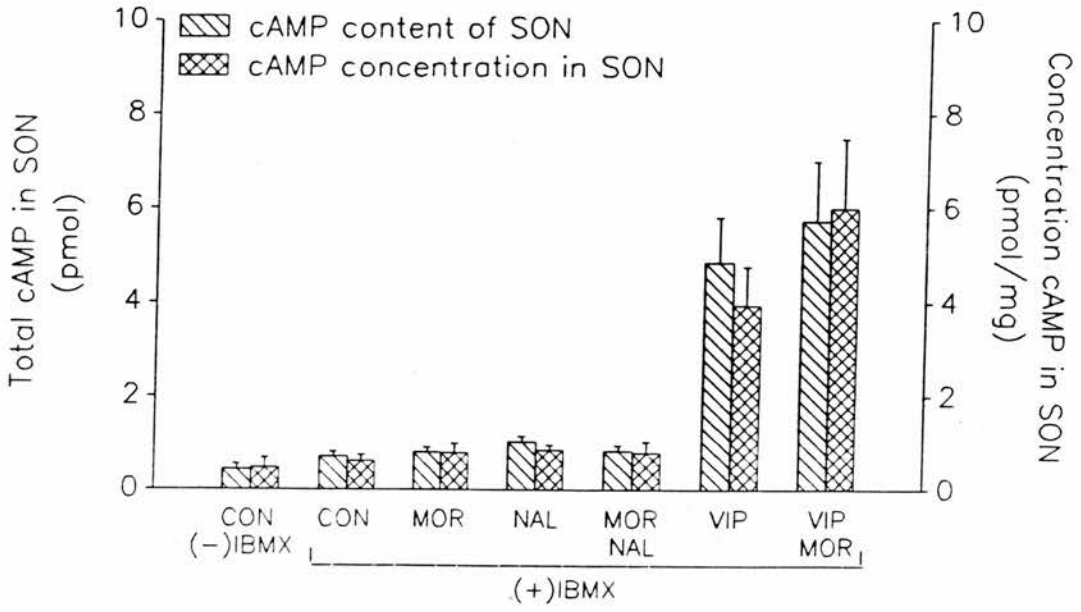


Figure 4.3.1.2. Experiment 1: cAMP-like immunoreactivity in SON taken from morphine-naive rats; effects of superfusion with morphine (MOR; 10^{-5}M), naloxone (NAL; 10^{-6}M) and VIP (10^{-6}M).

Data are derived from the previous table (Table 4.3.1.1.). Horizontal axis: treatment groups. Left vertical axis: cAMP content of SON (pmol; diagonal shading). Right vertical axis: cAMP concentration in SON (pmol/mg wet tissue; crosshatched shading).

SON were equilibrated in drug-free modified Yamamoto's solution for 90min including a 30min control period and were superfused with drug(s) diluted in the same solution as listed, for the following 30min (morphine 10^{-5}M, naloxone 10^{-6}M and VIP 10^{-6}M). Values represent the mean cAMP-like immunoreactivity present in the SON at the end of the protocol, when the tissue was removed from the chamber and collected for homogenisation and extraction of cAMP. Values are expressed both in terms of total content and as concentration of cAMP calculated from tissue wet weight.

There was no difference from control in the groups exposed to opiate and/or opiate antagonist (Kruskal-Wallis) but the tissues superfused with VIP responded with a dramatic increase in tissue cAMP content and concentration ($P=0.008$ and $P=0.012$ respectively, Wilcoxon test for independent samples). Likewise, those tissues superfused with VIP and morphine simultaneously also responded with a clear increase in cAMP content and concentration ($P=0.002$ and $P=0.002$ respectively, Wilcoxon test for independent samples). All tissues received bacitracin. IBMX was included where stated.

4.3.2. Experiment 1: OT/AVP release from SON taken from morphine-naive rats; effects of superfusion with morphine , naloxone and VIP.

Oxytocin was not reliably detected in superfusates.

Arginine vasopressin (AVP) was detected in all samples. AVP concentration in the absence and presence of IBMX with no drug treatment was 63.4 ± 19.3 and 41.8 ± 12.4 pg/30 min respectively. So, IBMX appears to have no effect on the release of AVP under these conditions.

Looking at these two control groups, there was a steady decline in AVP release with time. The total AVP released during the second 30 mins was 66.4 and 63.7% of that in the first 30mins in the absence and presence of IBMX. This fall in AVP output was reflected in the other treatment groups and in none of them did AVP release in the second 30mins, ie. in the presence of the treatment, reach 100% that in the first 30mins.

To obtain a more accurate measure of the profile of AVP release, a comparison was made of release over the first 30 mins and over the final 20 mins. Sample 4 was thought to be possibly a misleading component of the release of AVP in the second 30 mins due to the time lag for the drug solution to reach the chamber and to then equilibrate. So, the mean of the first three values and the mean of values 5 and 6 were compared. The index generated in this way also reflects the trend with time of a fall in output of AVP. None of the treatments resulted in deviation from the control index. So, in terms of AVP release, morphine 10^{-5}M alone, naloxone 10^{-6}M alone or both simultaneously had no effect on the release of AVP from vasopressin-secreting cells in the SON in vitro. VIP, which had a dramatic effect on tissue content of cAMP had no effect on AVP release in the nucleus (for summary, see Table 4.3.2.1.).

Table 4.3.2.1. Experiment 1: AVP-like immunoreactivity in SON taken from morphine-naive rats: effects of superfusion with morphine (10^{-5}M), naloxone (10^{-6}M) and VIP (10^{-6}M).

Treatment group	Total 1+2+3 (pg/30min)	Mean 1+2+3 (pg/10min)	Mean 5+6 (pg/10min)	Mean 5+6/ mean 1+2+3 (%)
a) control no drugs (+)bacitracin (-)IBMX	63.4 ± 19.3 (6)	21.2 ± 6.4 (6)	11.8 ± 4.7 (6)	58.0 ± 12.0 (6)
b) control No drugs (+)bacitracin (+)IBMX	41.8 ± 12.4 (9)	13.9 ± 4.1 (9)	11.36 ± 3.8 (8)	60.8 ± 15.8 (7)
c) (+)morphine 10^{-5}M (+)bacitracin (+)IBMX	109.0 ± 28.0 (8)	36.6 ± 9.3 (8)	23.0 ± 9.3 (8)	63.0 ± 11.9 (8)
d) (+)naloxone 10^{-6}M (+)bacitracin (+)IBMX	104.3 ± 43.7 (6)	34.8 ± 14.6 (6)	9.6 ± 3.9 (5)	48.2 ± 23.8 (5)
e)(+)morphine 10^{-5}M (+)naloxone 10^{-6}M (+)bacitracin (+)IBMX	114.6 ± 44.3 (5)	38.2 ± 14.6 (5)	23.0 ± 6.8 (5)	72.4 ± 20.8 (5)
f) (+)VIP 10^{-6}M (+)bacitracin (+)IBMX	59.4 ± 26.2 (5)	19.8 ± 8.7 (5)	14.6 ± 7.2 (5)	66.7 ± 12.6 (5)

g) (+)VIP 10^{-6}M (+)morphine 10^{-5}M (+)bacitracin (+)IBMX	96.9 ± 19.8 (6)	32.3 ± 6.6 (6)	18.3 ± 2.9 (6)	69.3 ± 13.5 (6)

Table 4.3.2.1. The sum of the individual values recorded for each SON (data not shown) for samples 1, 2 and 3 was calculated and the mean of these totals for each group expressed in the first column of figures as Total 1+2+3 (pg/30min). This gives a grand total of AVP release during the control period. Second, the mean of samples 1, 2 and 3 for individual SON was calculated and these then used to to calculate the mean release of AVP during an average 10min collection period for any one group. These data are expressed in the second column as Mean 1+2+3 (pg/10min). Third, the last procedure was applied to samples 4, 5 and 6 to derive the data expressed in the third column (pg/10min). Lastly, an index of release in the treatment period as compared with the control period was calculated from the individual data sets contributing to columns 2 and 3 (data not shown) and was expressed in the fourth column as a percentage. This is meant to quantify any change in AVP release that follows drug superfusion. There was no significant difference in AVP release between treatment groups and control (Kruskal-Wallis). All groups received bacitracin. IBMX was included where stated.

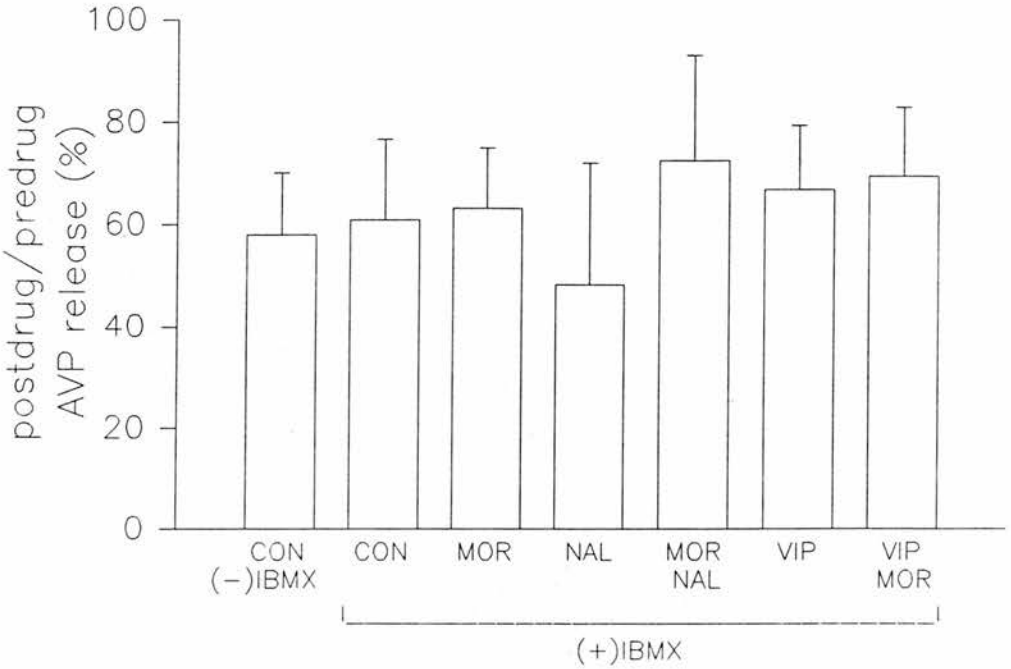


Figure 4.3.2.2. Experiment 1: AVP-like immunoreactivity in SON taken from morphine-naive rats: effects of superfusion with morphine (MOR; 10^{-5}M), naloxone (NAL; 10^{-6}M) and VIP (10^{-6}M).

Data are derived from Table 4.3.2.1.. Horizontal axis: treatment groups. Vertical axis: postdrug/predrug AVP release, mean percentage ± standard error of the mean SEM). The first 30min (sample periods 1, 2 and 3) was taken as the control period to assess basal release with any trend and during this period. Tissues were superfused with modified Yamamoto's solution alone and the control group went on to be superfused with drug-free modified Yamamoto's solution. The remaining groups were superfused for the last 30min with morphine (10^{-5}M), naloxone (10^{-6}M) or VIP (10^{-6}M) and for all groups, the index of AVP release produced by comparison of these two periods as listed in Table 4.3.2.1. was plotted. There was no significant difference in AVP release between treatment groups and control (Kruskal-Wallis). All groups received bacitracin. IBMX was included where stated.

4.3.3. Experiment 2: cAMP formation in the SON; effects of acute and chronic morphine

4.3.3.1. Experiment 2a: effects of acute morphine (10^{-4}M) on cAMP formation in SON from normal rats

cAMP was not consistently detectable in the superfusates.

In the tissue fragments superfused only with normal medium, mean (±SEM) cAMP content was 12.2 ±1.7 pmol (n=9) and in the tissue superfused with 10^{-4}M morphine, 14.0 ± 2.35 pmol (n=9), not significantly different (Table 4.3.3.3. and Figure 4.3.3.4.). There was no significant effect of morphine on tissue concentration of cAMP; in the control group this was 7.7 ± 1.3 pmol/mg tissue (n=9) and in the morphine superfused group 9.6 ± 1.2 pmol/mg tissue (n=8). In the control group the mean tissue sample weight was 1.66 ± 0.15mg and in the morphine group, 1.55 ± 0.14mg (see Table 4.3.3.3.).

4.3.3.2. Experiment 2b: Effects of naloxone (10^{-5}M) on cAMP formation in SON from i.c.v. morphine infused rats

In the tissue fragments superfused with morphine (10^{-5}M)-containing medium only, the mean ± SEM cAMP content was 13.45 ± 1.6 pmol (n = 6) and in the tissue superfused with naloxone-containing medium, the mean content was 15.5 ± 2.50 pmol (n = 6; not significantly different, Kruskal-Wallis). Similarly, there were no differences in the tissue concentrations of cAMP: these were 11.2± 1.8 pmol/mg in the control group and 14.9 ± 2.5 pmol/mg in the naloxone-treated group. The mean tissue weight in the control group was 1.23 ± 0.05mg and in the naloxone-treated group was 1.1 ± 0.11mg. (see Table 4.3.3.3.). The lower weights in this experiment compared with the first probably reflects more accurate trimming of the optic chiasm.

Table 4.3.3.3 Experiment 2: cAMP-like immunoreactivity in SON taken from morphine-naive and chronic i.c.v. morphine-treated rats; effects of superfusion with morphine (10^{-4}M/10^{-5}M) and/or naloxone (10^{-5}M).

Treatment group	Mean tissue weight (±SEM, mg)	Mean total cAMP (±SEM, pmol)	Mean cAMP/mg (±SEM,pmol/mg)
a) control (n=9) No drugs in vitro (-)IBMX	1.66 0.15	12.19 1.66	7.66 1.26
b) control (n=8) (+)morphine (10^{-4}M) (-)IBMX	1.55 ± 0.14	13.99 ± 2.35	9.62 ± 1.23
c) i.c.v. morphine (n=6) (+)morphine 10^{-5}M (-)IBMX	1.23 ± 0.05	13.45 ± 1.59	11.23 ± 1.77
d) i.c.v. morphine (n=6) (+)morphine10^{-5}M (+)naloxone 10^{-5}M (-)IBMX	1.10 ± 0.11	15.52 ±2.50	14.92 ±2.52

Table 4.3.3.3. Group size (n) is indicated with each group heading in parentheses. cAMP content and concentration are expressed as mean values ± standard error of the mean (SEM). There were no statistically significant differences between groups (Kruskal-Wallis).

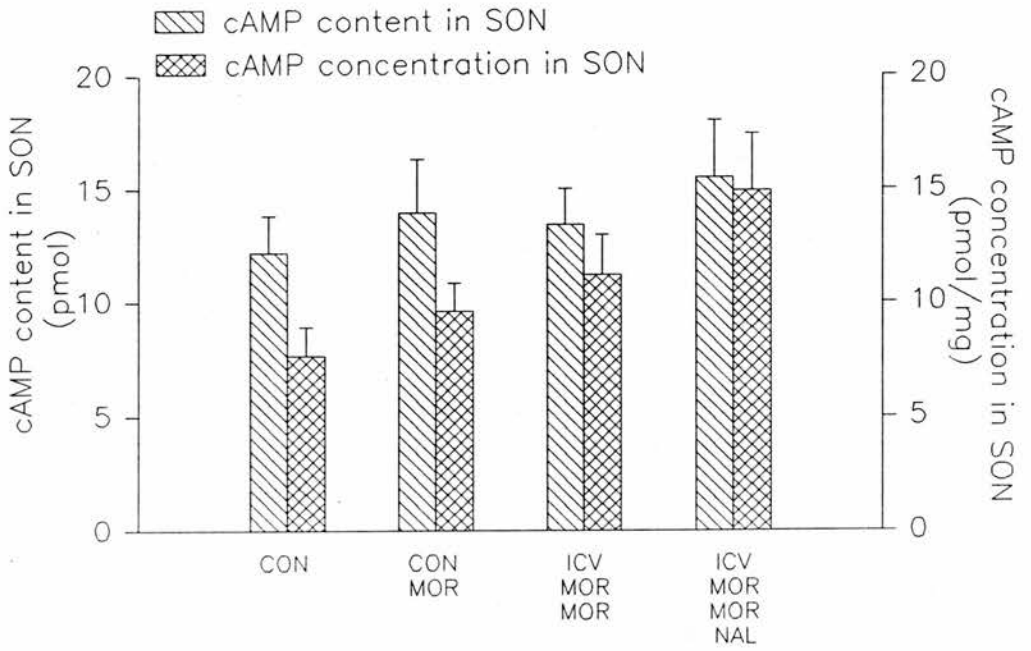


Figure 4.3.3.4. Experiment 2: cAMP-like immunoreactivity in SON taken from morphine-naive and chronic i.c.v. morphine-treated rats; effects of superfusion with morphine (MOR; 10-4M/10-5M) and/or naloxone (NAL; 10-5M).

Figure 4.3.3.4. Horizontal axis: treatment groups. Left vertical axis: cAMP content of SON (pmol; diagonal shading). Right vertical axis: cAMP concentration in SON (pmol/mg wet tissue; crosshatched shading). Treatment groups were CON: control group I, CON MOR: control group II, ICV MOR MOR: i.c.v. morphine-infused 'control' group I and ICV MOR MOR NAL: i.c.v. morphine-infused / acute naloxone-treated group (see Methods Section 4.2.4.4.).

SON were equilibrated in drug-free modified Yamamoto's solution for 90min including a 30min control period and were superfused with drug(s) diluted in the same solution, for the following 20min (morphine, 10^{-4}M for tissue taken from morphine naive rats or 10^{-5}M for tissue taken from chronic morphine treated rats; naloxone, 10^{-5}M). Values represent the mean cAMP-like immunoreactivity present in SON at the end of the protocol, when the tissue was removed from the chamber and collected for homogenisation and extraction of cAMP. Values are expressed both in terms of total content and as concentration of cAMP calculated from tissue wet weight. In no case was there a statistically significant difference between groups (Kruskal-Wallis).

4.3.4. Experiment 2: OT/AVP release from SON taken from morphine-naive and chronic morphine-treated rats; effects of superfusion with morphine and/or naloxone

OT was not consistently detectable in the superfusate.

There was a trend in AVP release with time in this preparation. The release over the first 30 mins exceeded that in the following 20 mins as indicated by the control group values: 2.78 ± 0.76 and 1.15 ± 0.63 pg/10min (mean ± SEM) for the pre- and post-treatment periods, a post-/pre-treatment index of 82.41%. The two 10min samples post-treatment were combined for comparison in this study with pre-treatment values to produce the index. As an early response of cAMP was expected, the two samples immediately after drug treatment commenced were taken for both cAMP and OT/AVP radioimmunoassay.

Comparison of AVP release between the groups revealed significant differences (P=0.03, Kruskal-Wallis). Superfusion with morphine at 10^{-4}M resulted in facilitation of AVP release in tissue taken from virgin morphine-naive rats. The index of post-/pre-treatment mean concentration of AVP was 205.5% and this was significantly different from control (P=0.04, Wilcoxon test for independent samples). So acute morphine treatment increased AVP output in SON magnocellular neurones in vitro (Table 4.3.4.1. and Figure 4.3.4.2.).

In contrast, SON taken from chronic morphine-treated rats did not show an increase in AVP release with time. There was no discernible change in the post-/pre-treatment index (62.0%), which was similar to control. Interestingly, the addition of naloxone along with acute morphine to the chronic morphine-treated preparation did not have any effect on AVP output which was similar to both untreated control and to the chronic morphine-treated tissue superfused with morphine (see Tables 4.3.4.1. and Figure 4.3.4.2.).

Table 4.3.4.1. Experiment 2: AVP-like immunoreactivity in superfusate of SON taken from morphine-naive and chronic morphine-treated rats: effects of superfusion with morphine (10^{-4}M/10^{-5}M) and/or naloxone (10^{-5}M).

Treatment group	Total 1+2+3 (pg/30min)	Mean 1+2+3 (pg/10min)	Mean 4+5 (pg/10min)	Mean 4+5/ mean 1+2+3 (%)
a) control No drugs in vitro (-)IBMX	7.72 ± 2.23 (8)	2.78 ± 0.76 (8)	1.15 ± 0.63 (8)	82.41 ± 49.61 (8)
b) control (+)morphine 10^{-4}M (-)IBMX	6.64 ± 1.74 (11)	2.31 ± 0.59 (11)	3.15 ± 0.37 (11)	205.5 ± 44.70 (10)
c) i.c.v. morphine (+)morphine 10^{-5}M (-)IBMX	12.98 ± 2.54 (6)	4.33 ± 0.85 (6)	2.00 ± 0.56 (6)	62.02 ± 32.50 (6)
d) i.c.v. morphine (+)morphine10^{-5}M (+)naloxone 10^{-5}M (+)IBMX	12.67 ± 3.75 (5)	4.15 ± 3/74 (5)	2.26 ± 0.75 (6)	57.6 ± 20.6 (5)

Table 4.3.4.1. The sum of the individual values recorded for each SON (data not shown) for samples 1, 2 and 3 was calculated and the mean of these totals for each group expressed in the first column of figures as Total 1+2+3 (pg/30min). This gives a grand total of AVP release during the 30min control period. Secondly, the mean of samples 1, 2 and 3 for individual SON was calculated and these then used to calculate the mean release of AVP during an average 10min collection period for any one group. These data are expressed in the second column as Mean 1+2+3 (pg/10min). Thirdly, the last procedure was applied to samples 4 and 5 to derive the data expressed in the third column (pg/10min). Lastly, an index of release in the treatment period as compared with the control period was calculated from the individual data sets contributing to columns 2 and 3 (data not shown) and was expressed in the fourth column as a percentage. This is meant to quantify any change in AVP release that

follows drug superfusion.

The groups differed significantly (P=0.03, Kruskal-Wallis). Superfusion with morphine (10^{-4}M) resulted in an increase in the release of AVP. Comparison of mean 1+2+3 with mean 4+5 revealed a significant change (P=0.04, Wilcoxon test for independent samples).

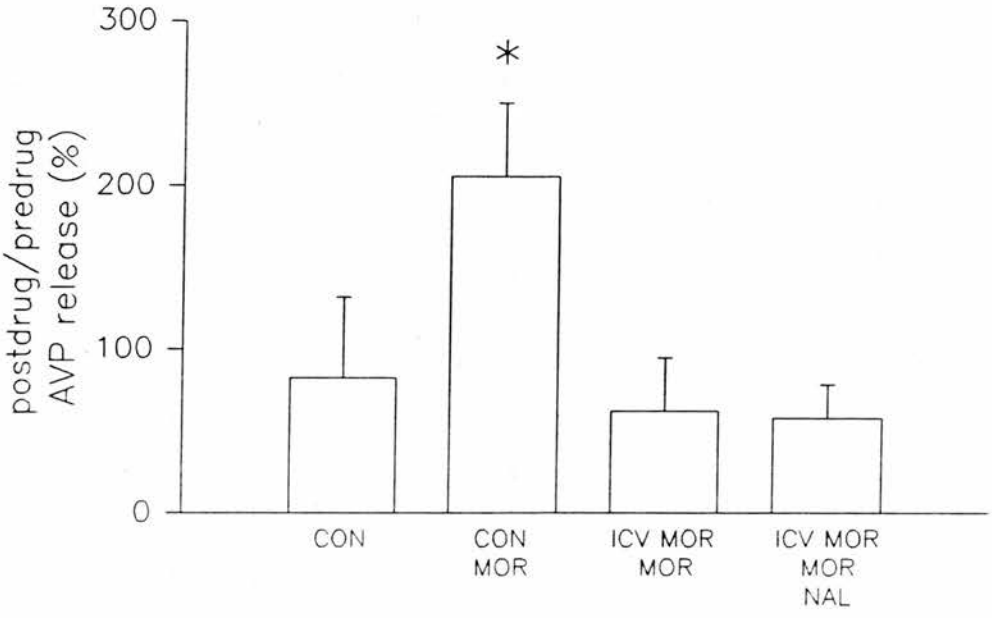


Figure 4.3.4.2. Experiment 2: AVP-like immunoreactivity in superfusate of SON taken from morphine-naive and chronic morphine-treated rats: effects of acute superfusion with morphine (MOR; 10^{-4}M/10^{-5}M) and/or naloxone (NAL; 10^{-5}M).

Figure 4.3.4.2. Horizontal axis: treatment groups. Vertical axis: post-drug/pre-drug AVP release, mean percentage ± standard error of the mean. Treatment groups were CON: control group I, CON MOR: control group II, ICV MOR MOR: i.c.v. morphine-infused 'control' group I and ICV MOR MOR NAL: i.c.v. morphine-infused / acute naloxone-treated group (see Methods Section 4.2.4.4.).
The first 30min (sample periods 1, 2 and 3) was taken as the control period to assess basal release and during this period tissues were superfused with modified Yamamoto's solution alone and the control group went on to be superfused with drug-free modified Yamamoto's solution. The remaining groups were superfused for the last 20min with morphine (10^{-4}M for tissue taken from morphine naive rats or kept in 10^{-5}M morphine throughout, for tissue taken from chronic morphine treated rats) and/or naloxone (10^{-5}M) and for all groups the index of AVP release produced by comparison of these two periods, as listed in Table 4.3.4.1. was plotted. Superfusion with morphine (10^{-4}M) resulted in an increase in the release of AVP ($P=0.04$, Wilcoxon test for independent samples).

4.4. DISCUSSION

4.4.1. Technical considerations

The protocol used in Experiment 1 differs from that used in Experiment 2 in three respects. The phosphodiesterase inhibitor IBMX (3-isobutyl, methyl xanthine) was included in the superfusing medium in order to preserve cAMP formed and therefore to amplify any changes due to drug treatment (Beavo et al, 1970). In Experiment 2 this was not done to limit the possible effects of accumulating cAMP itself on the physiology of the neurones in the SON. However, although measurable at low concentrations in the tissue, it proved impossible to detect cAMP under these conditions in the superfusate (which reflects cAMP release during the experiment) and there were no changes in tissue cAMP in Experiment 2. It was considered necessary to include IBMX in the protocol for Experiment 1.

The second change was the addition of the peptidase inhibitor bacitracin. It was excluded from Experiment 2 because of concern over general effects on a range of peptides known to be present in the SON, thus possibly altering the basal status of the neurosecretory cells. It is conceivable that the maintenance of higher than normal concentrations of OT itself may by a positive feedback mechanism alter the dendritic release of OT in the nucleus (Theodosis, 1985; Pow and Morris, 1989). Dynorphin too might be present in abnormally high concentrations with the implications that has for the control of OT-secreting neurones in the SON. To avoid these possible interactions therefore, bacitracin was omitted from the protocol in Experiment 2. The release of AVP was measurable but small in Experiment 2 but the release of OT was not reliably measurable. It was important to limit the possible breakdown of AVP and OT to make changes after drug treatment more pronounced, so bacitracin was included in the superfusate for Experiment 1. Comparison of AVP release over the first thirty minutes, in the absence of IBMX and in the absence and presence of bacitracin revealed that bacitracin certainly prevented the breakdown of basal AVP.

Total AVP released in the 30min control period in Experiment 2 was 7.72 ± 2.23 pg and in Experiment 1, 63.4 ± 19.3 pg, an increase of 721.2%. A similar rise in the concentration of dynorphin cannot be ruled out but OT was again not measurable above blank concentrations.

Third, the collection of a 50μl aliquot from the superfusate sample each 10mins for cAMP radioimmunoassay was not carried out for Experiment 1 and instead of following cAMP release, terminal tissue content and concentration were measured. All of the superfusate sample was therefore taken for OT and AVP radioimmunoassay.

It is questionable whether cAMP in the superfusate, if it could be measured, is a physiological measure of cAMP accumulation in the tissue. It would certainly be a more dynamic reflection of cAMP formation than the one measurement obtained at the end of the experiment of tissue content. However, it probably is not normally released by neurones and is presumably leaking from severed axons and somas in a non calcium-dependent way. Throughout the duration of the experiment one would expect the closing of cut processes to occur, thus restricting the leakage of cAMP from the neurones. The profile of cAMP 'release' would therefore not simply depend upon equilibration of intracellular cAMP with the extracellular fluid, but would depend upon the extent and rate of closure of cut processes. Nevertheless, large changes in intracellular cAMP should be reflected in the superfusate.

The difficulty in measurement of cAMP in the superfusate from Experiment 2 perhaps reflects rapid constriction of severed processes but measurement of the final tissue content is an alternative guide to any drug-induced change having occurred.

4.4.2. SON cAMP content and concentration in response to acute and chronic morphine exposure

cAMP formation is enhanced by a variety of neuropeptides including VIP which has been shown previously to potently stimulate cAMP formation in the SON and PVN in vitro (Redgate et al, 1986). Conversely, the opioids met- and leu-enkephalin

and β-endorphin inhibited the formation of cAMP under the same conditions (Redgate et al, 1986). Agonists at the κ-type opioid receptor do not stimulate adenylate cyclase (AC) in guinea pig striatal membrane (De Montis et al, 1986). Unfortunately a study using more receptor subtype selective agonists in the SON has not been done. Met-enkephalin has a preference for the δ-type opioid receptor as does leu-enkephalin (Kosterlitz, 1985) but the absence of the δ-opioid receptor subtype in the SON (Mansour et al, 1987; Tempel and Zukin, 1987; Mansour et al, 1988; Sumner et al, 1990) and the extremely low affinity of these agonists for the κ-opioid receptor (Kosterlitz, 1985) suggests that the inhibition of cAMP in the SON was mediated by the μ-opioid receptor subtype. Morphine has a preference for the μ-type opioid receptor with negligible affinity for the κ-type or δ-type opioid receptors (Kosterlitz, 1985). It was expected therefore that cAMP would be depressed with acute exposure to morphine. In neither Experiment 1 nor Experiment 2 was this the case. Morphine was completely without effect at moderate or high concentration.

cAMP has been implicated for some time in mediating the acute effects of opiates and by extrapolation the cellular changes that constitute tolerance and dependence (Collier and Roy, 1974). These experiments on neuronal cell membranes have been corroborated in cultured neuroblastoma x glioma cells, rich in opiate receptors (Klee and Nirenberg, 1974), which respond to acute exposure to opiates with a decrease in intracellular cAMP. During chronic opiate exposure AC activity returned to normal and removal of opiates led to a dramatic increase in AC activity, well beyond control levels (Sharma et al, 1975; Traber et al, 1975; Sharma et al, 1977.). The primary effect of inhibition of AC is a fall in the intracellular concentration of cAMP. cAMP-dependent protein kinase activity is thus reduced and many substrate proteins remain unphosphorylated so affecting cellular metabolism and perhaps neuronal excitability. In the chronic presence of opiates, the homeostatic response of the cell is presumably to re-establish the 'normal' rate of production of cAMP and so AC activity is enhanced. Upon withdrawal of the opiate, this hypertrophy of AC becomes

inappropriate and is reflected in the withdrawal syndrome. Here then is a possible cellular mechanism which could mirror the electrophysiological and behavioural parameters indicative of opiate tolerance, dependence and withdrawal.

However, these results have proved difficult to replicate in the CNS neurones known to have opiate receptors. Nestler and Tallman have suggested that this is partly accounted for by the heterogeneous nature of cell groups studied, rendering any change in AC difficult to detect (Nestler and Tallman, 1988). They looked instead at the homogeneous grouping of noradrenergic neurones in the locus coeruleus (LC) and studied the activity of cAMP-dependent protein kinase. Opiates inhibit the firing rate of LC neurones via G-proteins and a role for cAMP has been supported (Aghajanian and Wang, 1986) and questioned (North et al, 1987). LC neurones become tolerant to and dependent upon chronic opiates in terms of firing rate (Andrade et al, 1983). The study of Nestler and Tallman has shown an increase in cAMP-dependent protein kinase in both the particulate and the soluble fraction of LC, not seen in other brain regions examined. As the acute responses of these neurones to opiates is inhibition of AC (Duman et al, 1987) it is assumed that this would result in less cAMP and consequently less activated cAMP-dependent protein kinase. Thus the increase in cAMP-dependent protein kinase activity with chronic opiate may reflect an attempt to overcome the shortage of activated protein kinase.

The modulation of cAMP-dependent protein kinase by opiates could be mediated by μ- or κ-receptors, which predominate in the LC. Evidence from cultured neuroblastoma x glioma cells has implicated δ−receptors in the modulation of AC suggesting that the mechanism could involve any opiate receptor subtype. Indeed, in the 7315c pituitary tumour cell which has a population of exclusively μ−opiate receptors, the μ-selective agonist, Tyr-D-Ala-Gly-MePhe-Gly-ol (DAGO) inhibits AC. In cells exposed chronically to morphine (5 hours) and then exposed to [D-Ala^2-D-Leu^5]-enkephalin (DADLE) thought in this system to be acting through a μ−opioid receptor, AC activity was no longer inhibited indicating the development of tolerance

(Puttfarcken et al, 1988).

So, chronic exposure to opiates has been shown to modulate the cAMP system in some neurones.

In the light of this evidence for a role for cAMP in the manifestation of opiate induced tolerance, dependence and withdrawal, the inability to detect any change in the tissue content of cAMP in response to acute and chronic exposure to morphine and to acute superfusion with naloxone was perplexing, but indicates that cAMP need not be involved in altered electrical excitability of SON neurones after opiate.

AVP causes the accumulation of cAMP in punched out SON from hypothalamic slices (Abe et al, 1983) and cAMP is thus implicated in the transduction of the neuronal effects of AVP in the SON. There are two types of AVP receptor, one of which is independent of AC, the V1 receptor and the other which is linked to AC, the V2 receptor. The two appear to exist on central neurones (Abe et al, 1983). So, the content and concentration of cAMP in the SON in vitro might be influenced by the tissue concentration of AVP which although it remained unchanged throughout Experiment 1, was substantially raised during the superfusion of morphine in the Experiment 2. The cAMP content of SON treated with morphine was not different from control. If the effect of AVP is then to increase cAMP content in the SON (Abe et al, 1983) and the acute effect of morphine is to lower tissue content of cAMP (Puttfarcken et al, 1988), then the unexpected observation of no change against control could be accounted for.

Alternatively, the difficulty in measuring cAMP release in this nucleus in both series of experiments might be due to the heterogeneous cell population: OT- and AVP-secreting neurones which behave quite differently electrophysiologically and pharmacologically (Poulain and Wakerley, 1982). Others looking at heterogeneous groups of cells in the central nervous system have encountered similar difficulty in measuring cAMP (Satoh et al, 1976; Dafny et al, 1979). So it is perhaps optimistic to expect to see a clear cut response in terms of tissue content of cAMP in this group of

cells although cAMP might well play a pivotal role in mediating the effects of morphine, both acute and chronic.

However, a third possibility is that morphine might not be negatively coupled to AC in these cells and its effects on firing rate demonstrated here, might be produced by a different mechanism, as yet undefined. It follows therefore that the excitation seen in vivo when these neurones, once dependent upon morphine are withdrawn, may not be attributed to a rise in cAMP consequent to the hypothesised hypertrophy of AC. Another mechanism may be involved (see Chapters 3 and 5).

4.4.3. AVP and OT release

AVP release was increased in Experiment 2 in response to acute morphine (10^{-4}M) superfusion but was unchanged in response to naloxone in tissue derived from chronic morphine-treated rats; AVP release was also unchanged throughout Experiment 1. The withdrawal excitation of magnocellular neurones (and inhibition of some phasic, putative vasopressin secreting neurones) seen in vivo (Bicknell et al, 1988a) is not accompanied by a change in AVP release after naloxone within the SON in vitro.

It is interesting to note that although the tissue content of cAMP was dramatically increased with VIP there was no concomitant rise in AVP release. So, cAMP does not appear to play an important role in AVP release under these conditions. Although there is a reported accumulation of cAMP in response to AVP superfusion in punched out brain sections (Abe et al, 1983) the significance of cAMP accumulation evoked by other stimuli upon AVP release is obscure. Unfortunately, the relationship between OT release and cAMP accumulation remains unexplored due to the difficulty in measuring OT release in these experiments.

The increase in AVP release in response to morphine in Experiment 2 is unlikely to be a direct effect on AVP-secreting neurones. Opiates hyperpolarise central neurones (in locus coeruleus) directly at a somatic site (Pepper and Henderson, 1980) at the dendrites (Williams et al, 1982) and at the terminals (Nakamura et al, 1982) and these responses were consequent to activation of a μ-type opiate receptor (Williams and

North, 1984) which activates a potassium conductance (Williams et al, 1982). Opiates also inhibit hormone release from magnocellular neurosecretory neurones (Bicknell, 1985; Lincoln and Russell, 1986). There are however a variety of afferent inputs to the AVP-secreting cells some of which release inhibitory neurotransmitters (eg. GABA) which reduce AVP release. GABA is the major inhibitory input to these cells (Van den Pol, 1985; Randle et al, 1986; Theodosis et al, 1986; Buijs et al, 1987; Renaud, 1987; Sakaue et al, 1988) and electrophysiological investigation has revealed the inhibitory effect of GABA on these neurones in vivo (Arnauld et al, 1983) and in vitro (Randle et al, 1986). Presynaptic inhibition of this input by morphine would explain the unexpected increase in AVP release upon acute morphine superfusion. Agonists selective for the μ-type opiate receptor have been reported to act presynaptically to inhibit the activity of afferents to hypothalamic neurones (Loose and Kelly, 1989; Nikolarakis et al, 1989), although no evidence is available as yet concerning presynaptic opiate inhibition of a GABAergic input specifically in the hypothalalmus. However, there is evidence from the dentate gyrus and hippocampus CA1 region of the rat of opioid-mediated presynaptic inhibition of GABAergic inhibitory interneurones (Siggins and Zieglgansberger, 1981; Neumaier et al, 1988; Swearengen and Chavkin, 1989). So, this possibility is worth consideration.

The glia within the nucleus are also worthy of mention. They have a role in controlling the extracellular concentration of potassium (Kuffler et al, 1984). Glial cells in the posterior pituitary receive opioidergic synapses (Van Leeuwen, 1981) and are thought to have opiate receptors (Lightman et al, 1983b). Under conditions of increased hormone release they expose terminals of neurosecretory neurones and increase the area of contact with the capillary network (Tweedle, 1983). It is conceivable that morphine might act on the glial cells of the hypothalamic SON to alter their buffering function and thus create a more excitable environment due to a higher than basal extracellular concentration of potassium. Perhaps in a similar way to pituicytes in the posterior pituitary, the glia in the SON have a more direct

involvement than buffering the environment around the neurone: by plastic changes they might impair or improve the access of afferents to the cell bodies in the SON.

The fact that this result was not repeated in Experiment 1 could be due to the concentration difference for morphine; a 10-fold more concentrated solution was used in Experiment 2.

Others have attempted to measure hormone release from magnocellular neurosecretory neurones in vitro with varying degrees of success.

There is immunocytochemical evidence for the existence of axon collaterals from rat PVN neurones (Cobbett et al, 1983). SON neurones also appear to project axon collaterals into the region of the SON (Hatton et al, 1983). Although OT was known to increase the electrical activity of spontaneously-active OT neurones when injected intracerebroventricularly (Freund-Mercier and Richard, 1981) convincing morphological evidence for a pathway for release of OT local to the magnocellular nuclei was not available until 1985. An immunocytochemical study of OT and AVP distribution around and within the supraoptic nucleus of the rat revealed OT-like immunoreactivity not only on soma and dendrites, but also rarely on 'bouton-like' profiles mostly located in the dorsal confines of the nucleus (Theodosis, 1985). The profiles were not dendrites, judging by ultrastructural examination and contained 50nm diameter clear vesicles and occasionally dense-cored vesicles. Several of these immunoreactive profiles made synaptic contact with soma or dendrites also immunoreactive for OT. A more recent immunocytochemical study has demonstrated the competence of all parts of the plasmalemma of magnocellular neurones including dendrites and cell bodies in the release of OT and AVP within the nucleus (Pow and Morris, 1989). This could constitute the anatomical basis for the facilitatory pathway for OT regulation of its own release. So, AVP and OT are released within the SON. However, only OT is effective in facilitating its own release: AVP has no effect on OT release. AVP release is not modified by OT or AVP itself (Moos et al, 1984).

The above mentioned study also uncovered an interesting phenomenon: in

measuring OT and AVP release from isolated supraoptic and paraventricular nuclei Moos and coworkers showed that OT release was always substantially less than that of AVP. This result was not unprecedented as Chapman and coworkers had shown that in guinea pig SON, tissue content of AVP was 5 - 6 times greater than that of OT and that basal release of AVP was 10 - 80 times greater than that of OT (Chapman et al, 1983). In a later report (Mason et al, 1986), rat hypothalamic slices containing SON were sampled locally over the SON and a 50-fold difference in AVP vs. OT release was found. Given that OT and AVP are present in equimolar amounts in hypothalamic and pituitary stores in the rat, this was unexpected. The difference is possibly accounted for by the existence of OT in the nucleus in an incompletely processed form compared to AVP (Mason et al, 1986).

The difficulty in measuring OT release in vitro from the SON in the experiments described in this Chapter was not surprising in the light of the above findings. AVP release under basal conditions was 41.8pg/30min. This is substantially lower (21.7%) than that measured by Moos and coworkers using a similar protocol and SON in vitro from male rats: the equivalent of 192.6pg/30min for one nucleus. It follows that the more marginal release of OT reported by Moos and coworkers, 58.5pg/30min would be measured at approximately 12pg/30min. In the event, OT immunoreactivity was not detectable beyond that measured in blank medium samples. However, this did not prevent the examination of the primary objective, the effect of various treatments on the tissue content of cAMP.

These experiments have confirmed that AVP is released within the SON and that its release can be modified by acute morphine exposure. The significance of this finding is not clear.

There was no evidence for a connection between the hyperexcitation observed in SON OT neurones made tolerant to morphine and given naloxone and modulation of cAMP. Indeed, there was no change in tissue content of cAMP after acute or chronic morphine, even though it was greatly stimulated by VIP. The present data suggest that

AC plays an insignificant role in the adaptive events occurring during the development of opiate tolerance and dependence in OT neurones of the SON and is not involved causally in the changes in electrical activity in these neurones during exposure to morphine and naloxone. Indeed, the action of acute opiate upon firing rate is not correlated to changes in intracellular cAMP concentration in locus coeruleus neurones either (Karras and North, 1979), supporting this interpretation of the present results.

CHAPTER 5

MEASUREMENT OF CHANGES IN OPIATE RECEPTOR DENSITY IN RAT SUPRAOPTIC NUCLEUS AND MEDIAN PREOPTIC NUCLEUS AFTER CHRONIC MORPHINE TREATMENT: A QUANTITATIVE AUTORADIOGRAPHIC STUDY.

5.1. INTRODUCTION

Opioid receptors can be subdivided into three widely accepted categories, μ, δ and κ. The existence of subtypes of receptor was postulated to explain paradoxical effects of nalorphine in man (Martin, 1967). Opioid receptors have distinct distributions in the brain (Mansour et al, 1988), different affinity for various opioid ligands both endogenous and exogenous (Gilbert and Martin, 1976; Kosterlitz, 1985), different post-receptor cellular effectors and effects (see Chapter 3), different acute functional profiles (Redmond and Krystal, 1984) and different withdrawal syndromes (Redmond and Krystal, 1984). Opioid receptors and endogenous ligands are present in or close to the supraoptic nucleus (SON) where oxytocin (OT)-secreting neurones have their cell bodies (Clark et al, 1986; Mansour et al, 1986; Tempel and Zukin, 1987; Mansour et al, 1988; Sumner et al, 1990) and opioids are known to modulate both OT and vasopressin secretion (Bicknell and Leng, 1982) although the evidence for a clear interaction with OT neurones both at the level of the cell bodies and at the terminals in the posterior pituitary is more compelling than that for the vasopressin system (Muhlethaler et al, 1980; Pittman et al, 1980; Bicknell and Leng, 1982; Arnauld et al, 1983; Wakerley et al, 1983; Zhao et al, 1988b; Leng and Russell, 1989; Inenaga et al, 1990; Leng et al, 1990). Both morphine and the κ-opioid receptor agonist, U50,488H (*trans*-(±)-3,4-dichloro-*N*-methyl-*N*-(2-[1-pyrrolidinyl]cyclohexyl) benzeneacetamide, methane sulphonate salt) acutely change the electrical activity of OT neurones (Pittman et al, 1980; Wakerley et al,1983; Pumford et al, 1987; Russell et al, 1989b; Inenaga et al, 1990; Leng et al, 1990) recorded both in vivo and in vitro in the hypothalamic slice. Morphine, when given chronically by i.c.v. infusion causes tolerance in mechanisms controlling the electrical activity of OT neurones and also the secretion of OT into the peripheral blood (Bicknell et al, 1988a; Rayner et al, 1988; Sumner et al, 1989; Leng et al, 1990.). The development of tolerance to chronic i.c.v. morphine is always accompanied by dependence in the SON OT system and this is

manifested by the hyperexcitation of OT neurones and the hypersecretion of OT after the opiate antagonist, naloxone (Bicknell et al, 1988a; Rayner et al, 1988). This withdrawal response is partly mediated through the region anterior and ventral to the third ventricle (see Chapter 6), the AV3V region which is composed of the median preoptic nucleus (MPN), the organum vasculosum of the lamina terminalis (OVLT) and connections from the subfornical organ (SFO). The AV3V region is important in the maintenance of normal body fluid balance and its ablation impairs the normal response to a hyperosmotic challenge (Johnson and Buggy, 1978; Brody and Johnson, 1980; Blackburn et al, 1987; Russell et al, 1988). The AV3V region projects to the SON and contains axons coursing down to the SON from the SFO (Miselis, 1981; Renaud et al, 1983; Lind et al, 1985; Swanson and Lind, 1986). Although OT neurones in the SON have been shown to be osmosensitive in the absence of any synaptic input they are only fully osmoresponsive if the input from the AV3V region is intact (Russell et al, 1988). However, ablation of the AV3V region does not impair the activity of OT neurones during other unrelated processess such as milk-ejection in response to the suckling stimulus or parturition (Russell et al, 1988).

Morphine binds with a high relative affinity to the μ-type opioid receptor and is thought to act almost exclusively at this site. In Chapter 2 of the Thesis the possibility of tolerance to chronic i.c.v. morphine at the μ-opioid receptor resulting in heterologous tolerance at the κ-opioid receptor was examined (Leng et al, 1990). There was no evidence of heterologous tolerance in terms of the electrical activity of OT neurones recorded in vivo.

Using agonist ligands which are highly receptor subtype-selective, to displace the binding of the broad spectrum radioligand ([^{3}H](-)bremazocine) at the μ- and δ-opioid receptors, κ-opioid receptor binding in the SON was measured - this has not been quantified to date. No displacers were used in the MPN where, using the broad spectrum radioligand, [^{3}H]etorphine, the MPN was probed for opioid receptors without attempting to distinguish subtypes. Opioid receptor binding has not been

localised to MPN in studies performed to date.

Using the same technique, the question of whether receptor density in SON or MPN was influenced by chronic i.c.v. morphine infusion was investigated, as receptor density changes might account at least in part for desensitisation after chronic opiate. Additionally, whether the electrophysiological results described elsewhere in this Thesis (Chapter 2), which indicate no cross-tolerance between the μ- and κ-opioid receptors in terms of the electrical activity of OT neurones (receptor sensitivity), were borne out in terms of κ-receptor density in the SON was investigated. I have gone on in the Discussion section to speculate on possible mechanisms of regulation of opioid receptor density.

This work has been published (Sumner et al, 1990).

5.2. METHODS

5.2.1. Tissue

Brain sections were derived from two groups of rats: the control group was composed of rats (n=6, mean ± SEM body weight=288.8 ± 8.7g) taken five days before decapitation and, under ether anaesthesia, implanted with an i.c.v. infusion assembly driven by a subcutaneously-implanted osmotic minipump (Alzet 2001) containing sterile pyrogen-free distilled water. For 5 days the infusate was delivered into a lateral cerebral ventricle at 1μl/h.

The rats in the morphine-treated group (n=6, mean ± SEM body weight=274.8±5.1g) were taken 5 days before decapitation and under ether anaesthesia fitted with an i.c.v. infusion assembly, as above, but containing three sequentially increasing concentrations of morphine sulphate separated by 1μl air bubbles: 10μg/μl, 20μg/μl and finally 50μg/μl each delivered over a 40h period at 1μl/h. (The details of this procedure are given in Chapter 1).

On the day of experiment rats prepared as above were decapitated, the brain removed and blocked coronally, including tissue 1mm anterior to the optic chiasm through to the posterior limit of the mammillary bodies. Blocks were immediately frozen onto a pre-cooled layer of Tissue-Tek OCT compound (Miles Laboratories) on a cold cryostat chuck standing on dry ice. Blocks were stored in the freezer at -20°C before sectioning was carried out again at -20°C by cryostat (Bright Instrument Co. Ltd. Cambs., model FS/FAS/M). 10μm sections were thaw-mounted onto acid-cleaned, chrome alum-gelatine-subbed slides. Four sections were taken from each rat, containing SON and four containing MPN, for each incubation category. Mounted sections were stored at -20°C for up to 3 weeks before the next stage, incubation.

5.2.2. Ligands

The broad spectrum radioligands which were to be displaced by receptor subtype-selective ligands were [^{3}H]etorphine (50Ci/mmol which is equivalent to

1.85TBq/mmol, Amersham) and [^{3}H](-)bremazocine (21.3Ci/mmol which is equivalent to 0.79TBq/mmol, NEN). Highly receptor subtype-selective ligands were used as competitive displacers of these two broad spectrum radioligands to create conditions under which one receptor subtype could be isolated for measurement. For displacement from the μ-receptor, Tyr-D-Ala-Gly-MePhe-NH$(CH_2)_2$OH, (DAGO, Cambridge Research Biochemicals) and for displacement from the δ-receptor, [D-Pen^2,D-Pen^5]-enkephalin, (DPDPE, Sigma Chemical Co.). Naloxone hydrochloride (Sigma Chemical Co.) was used to competitively antagonise all binding.

5.2.3. Incubation procedure

The combinations of radioligand and displacers is summarised in Table 5.2.4.4. Total binding to opioid receptors was measured using a broad spectrum [^{3}H]-ligand (2nM) alone. Measurement of receptor subtypes was achieved using medium containing two unlabelled receptor subtype selective displacer ligands (2μM each) together with the [^{3}H]-ligand, in order to competitively displace the [^{3}H]-ligand from all but one of the receptor subtypes. The concentration of displacer was chosen after pilot experiments revealed no further displacement of [^{3}H]-ligand at concentrations higher than 2μM.

Pilot experiments showed more binding by [^{3}H](-)bremazocine than by [^{3}H]etorphine in brain sections incubated with DAGO and DPDPE. For this reason, measurement of κ-receptor binding was performed using [^{3}H](-)bremazocine as the broad spectrum ligand rather than [^{3}H]etorphine.

The details of this procedure for the use of [^{3}H]ligands have been optimised by others (Bunn et al, 1985). Preincubating and incubating media were made up in double-distilled, deionised water. Pilot experiments compared the effectiveness of the preincubation over a range of time, 0 to 90min for two protocols, one using [^{3}H]etorphine and the other [^{3}H](-)bremazocine as the broad spectrum radioligand.

5.2.3.1. Preincubation using [^{3}H]etorphine

[^{3}H]etorphine was used as the radioligand to probe for opioid receptors in the MPN and the following protocol was used (see Bunn et al, 1985). Sections were preincubated for 15min at room temperature in 0.05M Tris-HCl buffer, pH 7.4, containing 0.1M NaCl and 50μM Gpp(NH)p (5'-guanylylimido-diphosphate; to detach bound endogenous ligands). 40μl of preincubating solution was applied as a puddle to each section which was completely immersed. Slides were kept in moist chambers during the preincubation period after which the puddles of preincubating medium were tapped off the slides and replaced with incubating medium containing [^{3}H]etorphine ± displacer. As we looked just for overall binding to opioid receptors in MPN and did not attempt to identify the subtypes, the only displacer used was the opioid antagonist, naloxone. The incubation categories were then, 1. [^{3}H]etorphine alone (total opioid receptor binding), 2. [^{3}H]etorphine plus naloxone (specific opioid receptor binding) and 3. no [^{3}H]etorphine or displacers (chemographic control). The sections were than incubated for 1h at room temperature after which the incubating medium was tapped off the slides and the slides in racks gently immersed in 4 consecutive ice-cold buffer washes (0.05M Tris-HCl, pH 7.4, containing 0.19M sucrose) for 1min each wash. Finally, sections were rinsed in ice-cold double-distilled, deionised water to remove salts and dried in air. Sections were then stored dessicated in light-tight boxes until autoradiography, within the next 48h. Control and treatment pairs were processed identically and simultaneously.

5.2.3.2. Preincubation using [^{3}H](-)bremazocine

[^{3}H](-)bremazocine was used as the radioligand to probe for κ-opioid receptors in the SON and the following protocol was used (see Brady and Herkenham, 1987). Sections were preincubated for 15min at 0^{o}C in 0.015M potassium phosphate buffer, pH 7.4, containing 0.15M NaCl and 0.1% BSA (bovine serum albumin). Each section was immersed in a 40μl puddle of the preincubating medium and slides kept in a moist chamber during preincubation. After 30min, the puddles were tapped off the

sections and replaced with the same volume of incubating medium, containing [^{3}H](-)bremazocine ± displacers. Because we were looking for specific binding to the κ-subtype of opioid receptor there were 4 incubation categories: 1. [^{3}H](-)bremazocine alone (total opioid receptor binding), 2. [^{3}H](-)bremazocine plus naloxone (specific opioid receptor binding), 3. [^{3}H](-)bremazocine plus DAGO and DPDPE (specific κ-opioid receptor binding) and 4. no [^{3}H](-)bremazocine or displacers (chemographic control). The sections were then incubated for 3h at 0°C after which the incubating medium was tapped off the slides and the slides mounted into racks for immersion into 4 successive baths of ice-cold buffer wash (0.05M potassium phosphate buffer, pH 7.4, without any additives), 1min each wash. Finally sections were rinsed in ice-cold double-distilled deionised water to remove salts and dried in air. Sections were stored desiccated in light-tight boxes until autoradiography, within 48h. Control and treatment pairs were processed together simultaneously.

5.2.4. Control incubations

5.2.4.1. Naloxone control

One set of control incubations contained naloxone hydrochloride (2μM) together with [^{3}H]ligand (2nM), to confirm the nature of the receptors detected as opioid receptors. Naloxone at up tp 20μM did not further reduce [^{3}H]ligand binding in pilot experiments.

5.2.4.2. Naloxone plus displacers control

A second control series of sections was incubated with [^{3}H]ligand plus all 3 displacers to control for any possible unexpected interaction between naloxone and the [^{3}H]ligand other than competition for opioid binding sites. This configuration of ligands reduced naloxone binding and was used to assess the degree of specific binding to opioid receptors.

5.2.4.3. Chemographic control

A third group of control sections was incubated in an identical manner to test sections but in the absence of [^{3}H]ligand, to test for chemographic artifacts. Because

clusters of silver grains can occur as the result of interaction between tissue constituents, or chemical components of the incubation medium with the photographic emulsion, thus resulting in very reproducible false positives, these controls are essential.

Table 5.2.4.4. Incubation protocol

	Alone (all opioid receptors)	+ 2μM naloxone (specificity control)	No radioligand (chemographic control)	+ 2μM DAGO + 2μM DPDPE (κ)
+ 2nM [^{3}H]etorphine **MPN**	+	+	+	-
incubation code:	**E**	**EN**	**EC**	
+ 2nM [^{3}H](-)bremazocine **SON**	+	+	+	+
incubation code:	**B**	**BN**	**BC**	**BK**

5.2.5. Autoradiography

Sections were brought to room temperature and apposed to 'Hyperfilm'-^{3}H (Amersham) keeping slides from control/treatment pairs of rats, similarly incubated, adjacent. A 5μm section of a tritium standard (^{3}H-microscales, Amersham) was placed on each sheet of 'Hyperfilm' . The sheets of film with attached slides were then sandwiched between mirror-coated glass plates, held in place with adhesive tape, enclosed in light-tight wrapping and left to expose at 0-4°C in the dark.

The exposure period of 9 weeks was determined empirically to produce measurable grain densities over tissue sections which were below saturation, in all positive incubations.

After exposure, the films and slides were brought to room temperature and the slides removed for later histological preparation. The films were developed at 18°C in

Kodak D19 developer (5min), dip-rinsed in tap water, fixed in Ilford Hypam rapid fixer (two changes, 5 min each; 1:4 fixer:tap water), rinsed for 30min in running tap water and dried in air.

The sections were fixed for 10min in acetic acid: absolute ethanol: commercial formalin (1:17:2 by volume), washed in running water for 5min, dehydrated through a series of alcohol baths, 2-3min in each, placed in a xylene bath for 10min and rehydrated through alcohol baths in reverse. After a 5-10min wash in running water, sections were stained for 15min in 1% Cresyl Fast Violet, washed, dehydrated, in absolute alcohol, cleared in xylene and mounted in DPX.

5.2.6. Quantitative analysis of autoradiographs

Pieces of film corresponding to the apposed slide were cut out and attached to clean microscope slides with adhesive tape. Autoradiographs were paired with the stained sections and examined under a binocular dissecting microscope (Wild M3, magnification x16) to identify the SON and MPN.

The boundaries of the SON and MPN were outlined by scoring the photographic emulsion with a fine needle under the dissecting microscope. Silver grain density was then measured using a Joyce-Loebl μMagiscan image analysing computer with video input from a Philips black and white Video 40 camera (with a Newvicon tube) mounted on a Vickers M17 microscope. Microscope magnification was x10 (objective) and x1.6 (intermediate lens); the video monitor scale factor was 1.1878μm per pixel. A rectangular counting frame (201.63 x 133.04μm) was defined which was smaller than the area of the average SON so that measurements could be selectively taken over the dorsal SON and over the area of the MPN.

5.2.6.1. Expression of results as mean grain density

Silver grain density was calculated as, total area of silver deposit / total area of the counting frame. A non-tissue background count was made immediately after each tissue count, over an immediately adjacent region of film and subtracted from the tissue count. So, for each rat, mean grain density was determined for SON or MPN in each incubation category. On each sheet of film, 3 measurements were taken over the standard strip and 3 background measurements taken at the same time from adjacent film. The background counts were subtracted from the standard counts and these 3 values averaged to produce a mean standard grain density with which to compare the tissue counts. The specific binding to opioid receptors was calclated by subtraction of mean grain density over the corresponding naloxone sections, if they were significantly above background (Student's paired t-test).

Comparison of mean grain densities, thus calculated for each incubation category, was made between the i.c.v. vehicle-infused and i.c.v. morphine-infused rats by paired t-test, as they had been processed in pairs throughout.

5.2.6.2. Expression of results as absolute values of specific [^{3}H]ligand binding (fmol [^{3}H]ligand bound/mg tissue)

Mean grain density values (naloxone control counts not yet subtracted) were then transformed into absolute values. Grain density was measured in triplicate over each step of the standard scale, with backgounds subtracted as already described. A mean standard curve of grain density vs. radioactivity could then be constructed. This was expressed in terms of its brain-grey matter-tissue equivalent using calibration information provided by Amersham together with the standard scales (calibrated by Geary and Wooten for Amersham). The curve was described best by the power regression function $y=A \times x^B$

where, y=mean grain density of a given step on standard scale

x=radioactivity, converted to the tissue equivalent value

A=the intersect on the y axis and

B=the intersect on the x axis

of the plot of radioactivity vs. mean grain density for the steps of the standard scale measured. The mean standard curve thus constructed for SON and MPN fitted to the above power function where, $y=0.27 \times x^{0.23}$ $r=0.97$

where r=regression coefficient for the curve.

Tissue radioactivity values (nCi/mg of tissue, x), were then derived from the mean grain densities (y), using the above formula and were then converted to fmol of [^{3}H]ligand bound per mg of tissue, by reference to the specific activity of [^{3}H]ligand (see earlier). Specific binding to opioid receptors was obtained by subtracting binding remaining in the presence of naloxone at this stage. The film does not respond in a linear way in terms of the development of silver grain deposits upon exposure to radioactivity. With this in mind, the mean grain densities in the experimental sections and in the naloxone control sections were both transformed to absolute values, as outlined above, before the subtraction of the naloxone control value to obtain specific [^{3}H]ligand binding in fmol per mg tissue. I.c.v. morphine and control group mean [^{3}H]ligand binding values were compared by Student's t-test.

5.3. RESULTS

5.3.1. Supraoptic nucleus

Examples of autoradiographs obtained are displayed in Figure 5.3.1.3..

5.3.1.1. Mean grain density

Specific binding (naloxone-displaceable) was over 97% total binding in SON tested with [^{3}H]ligand alone. There was no significant difference between the i.c.v. vehicle-treated and i.c.v. morphine-treated rats in terms of mean silver grain density (corrected for non-specific binding) in the B category ([^{3}H](-)bremazocine alone) or in the BK category ([^{3}H](-)bremazocine + DAGO + DPDPE) for SON, indicating no change in the density of opioid receptors detected by [^{3}H](-)bremazocine and more precisely, κ-opioid receptors in the SON after chronic morphine treatment (see Table 5.3.3.).

5.3.1.2. Absolute density of [^{3}H]ligand binding

There was no discernable difference in total [^{3}H](-)bremazocine binding (total opioid receptors) or in selective binding to κ-receptors expressed as fmol [^{3}H](-)bremazocine bound per mg tissue after chronic morphine. This outcome mirrors that already seen in terms of mean grain density (see Table 5.3.3.).

5.3.2. Median preoptic nucleus

Examples of autoradiographs obtained are displayed in Figure 5.3.2.3..

There was a significant reduction of 31% in the mean silver grain density, measured in the MPN after chronic morphine treatment, indicating a lower overall density of opioid receptors in the MPN (see Table 5.3.3.).

5.3.2.1. Absolute density of [^{3}H]ligand binding

[^{3}H]etorphine binding was diminished by 77% expressed as fmol [^{3}H] (-)bremazocine bound per mg tissue (see Table 5.3.3.).

Table 5.3.3. Mean silver grain densities and absolute specific binding of [^{3}H]ligand (mean ±SEM) for SON and MPN in three incubation categories

Brain region	Incubation code	I.c.v. vehicle-treated group (n=6)	I.c.v. morphine-treated group (n=6)	Comparison of groups with paired t-test
SON	B	0.32±0.02 (125.7±35.0)	0.27±0.03 (70.9±24.8)	n.s.d.
	BK	0.32±0.01 (130.4±25.6)	0.30±0.02 (95.5±16.0)	n.s.d.
MPN	E	0.36±0.01 (76.2±9.8)	0.25±0.02 (17.5±5.2)	P<0.05

Data are expressed as μm^2 of silver deposit per μm^2 of field; corrected for non-specific binding; data in parentheses are specific [^{3}H]ligand binding expressed as fmol ligand bound per mg tissue; n.s.d., not statistically significant.

Figure 5.3.1.3.(overleaf) κ-opiate receptor binding in the supraoptic nucleus
Autoradiographs a., b. and c. represent binding categories B (total [^{3}H] (-)bremazocine binding), BK ([^{3}H] (-)bremazocine + displacers, DAGO and DPDPE) and BN ([^{3}H] (-)bremazocine + naloxone) respectively. The images become less intense from a. to c. due to the the progressive displacement of [^{3}H] (-)bremazocine from μ- and δ-opioid receptors by the displacers and in c., from all specific opiate receptor binding by the opiate antagonist, naloxone. The SON lies between the solid triangles, at the outer edge of the optic chiasm, which forms the ventral border of the image. The density of the image over the SON is less than that produced in other brain regions such as the caudate putamen, where dense spots can be seen, or the thalamus.

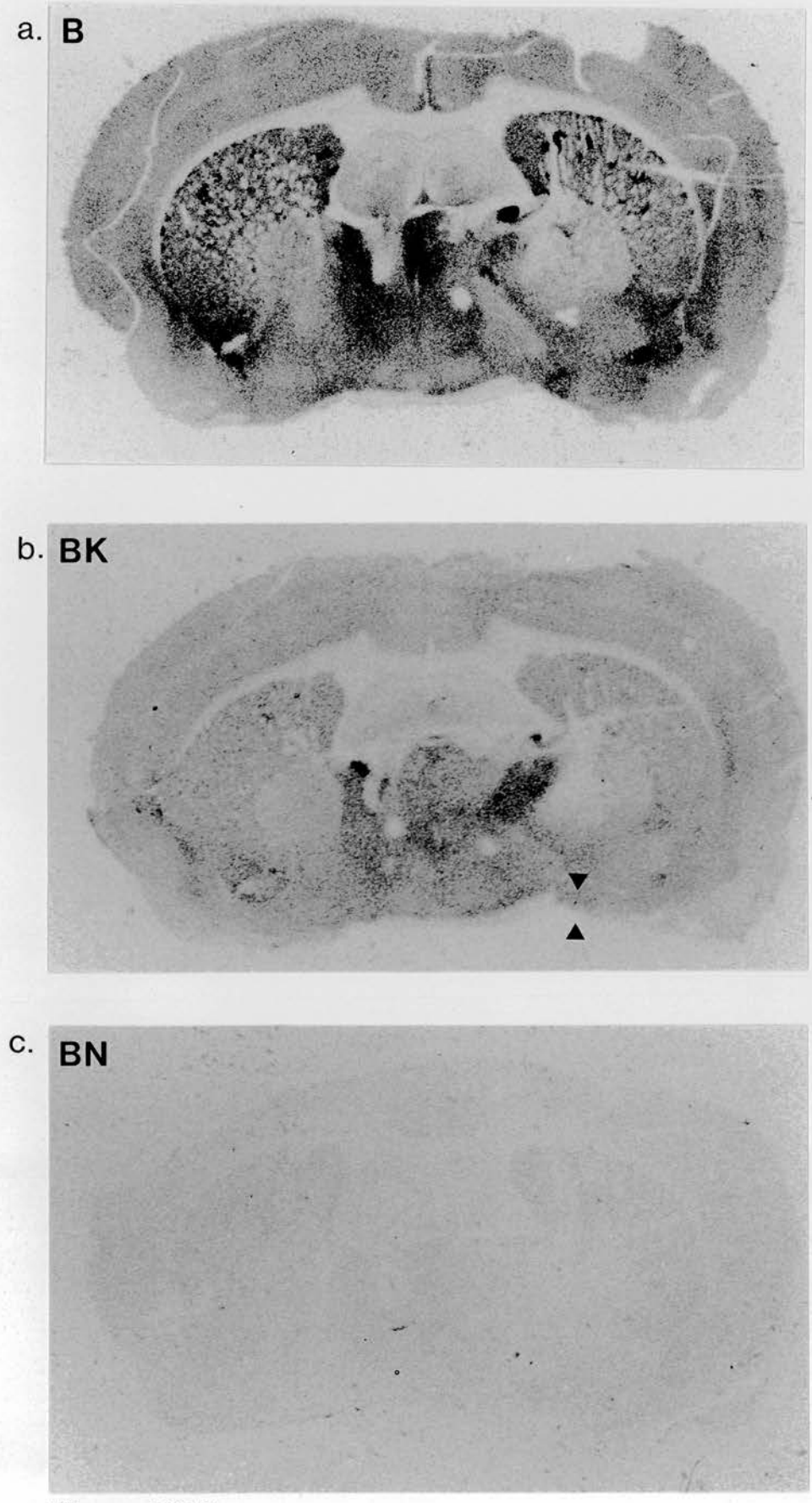


Figure 5.3.1.3.

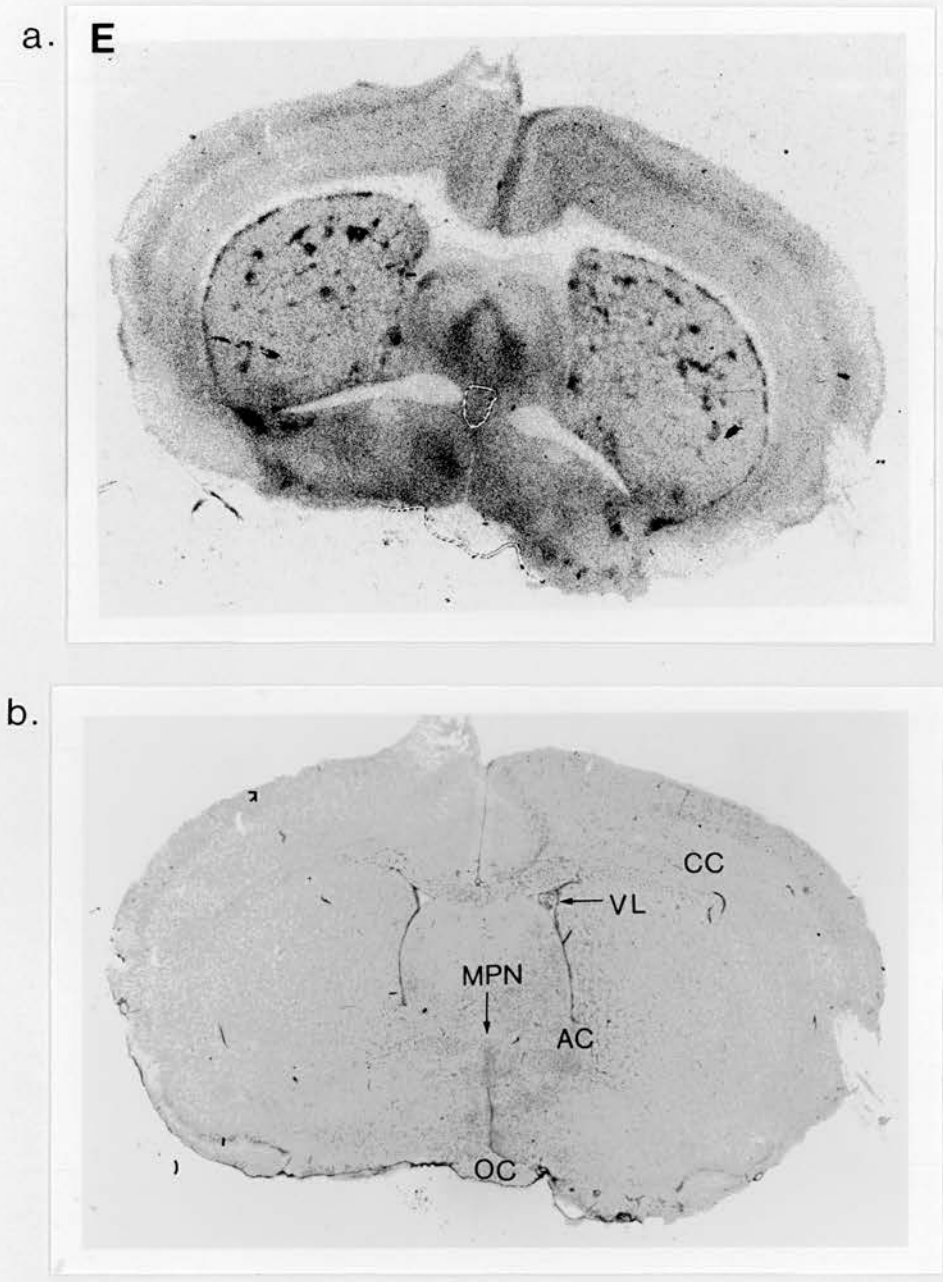


Figure 5.3.2.3. Opiate receptor binding labelled by [^{3}H]etorphine in MPN

Figure 5.3.2.3.a represents a typical autoradiograph depicting opiate receptor binding labelled with the broad spectrum opiate agonist radioligand [^{3}H]etorphine. The limits of the MPN and the optic chiasm have been scratched upon the photographic emulsion, using the equivalent tissue section (b.) for guidance to enable accurate measurement of grain density. The MPN lies medially, between the anterior commisure in this example.

Abbreviations: MPN: median preoptic nucleus; OC: optic chiasm; AC: anterior commisure; CC: corpus callosum and VL: lateral ventricle.

5.4. DISCUSSION

5.4.1. Technical considerations

Hyperfilm-^{3}H is designed to detect β-radiation from a tritium source primarily and is designed without an anti-scratch layer over the film emulsion to permit the closest apposition of tissue to film. Because β-particles will scatter on passing through film emulsion, Hyperfilm-^{3}H is a single-coated film resulting in a shorter path length travelled by the β-particles and consequently less scattering. This results in high resolution detection of radioactivity. However, a balance needs to be found. Although a thin section results in good resolution, variation in section thickness is more of a problem than with thicker sections and as quantification of these data was important, something by way of resolution was sacrificed for the sake of accuracy in measurement. It is only radioactivity in tissue apposing the film to a depth of about 1.5μm that contributes to the formation of the latent image. Emission of radioactivity from deeper within the section is likely to be absorbed within the tissue itself. Although different components of the tissue will absorb radioactivity to different degrees according to their densities, and thus will cause the development of an image that reflects a distortion of the true quantity of radioactivity in the tissue. This does not cause difficulties in these experiments however as like areas of tissue were compared throughout the study and silver grain density was not measured at a high enough resolution to have to apply compensating factors to account for differential absorption of β-emissions by cell structures.

Because Hyperfilm-^{3}H is so sensitive to ionising radiations, it can also suffer fogging during storage and exposure due to silver grain activation by natural background radiation; cosmic rays, γ-emitters in air, building materials etc., exposure to light or to exhausted darkroom chemicals. Although during exposure, the films were protected with several layers of wrapping, one of which was aluminium, and during darkroom procedures only recommended safe light sources were used, all

environmental radiation sources could not be eliminated, such as γ- and X-radiation both of which will penetrate aluminium. Thus some unavoidable background fogging was detected on all films.

Temperature increases the development of fogging and the recommended temperatures were followed during all procedures to reduce this potential problem.

Pressure was evenly applied to the sheets of film with slides mounted but whilst the pressure applied should be great enough to bring the section and film close together thus improving the definition of silver grain development and the accuracy of the distribution of radioactivity, it should not be so great as to cause artefactual silver grain activation due to excess pressure.

Transfer of static electricity to the film during handling can cause artefactual activation of silver grain. Stresses in the film can cause distortions in the distribution of silver grain, so the film should be handled with care. Finally, positive chemography, interaction between the film and tissue constituents, can cause an aggregation of silver grains.

All of the above result in positive artefacts, but there are also negative artefacts. These include moisture either on the slides or on the skin coming into contact with the film during exposure which can fade the latent image, as can negative chemography by an oxidative chemical reaction.

These problem areas were kept in mind and eliminated as far as possible by the use of careful technique and appropriate controls.

5.4.2. Quantification of results

There should be if not stoichometry, at least a proportionality between the amount of radioactivity and the number of silver grains, in order that the autoradiographic image can be quantified. The safest way to control for this is by the use of radioactive standards of known activity against which to measure the tissue image. Any non-linearity in the response of the emulsion, which can occur at either extreme is thus taken into account. The present results were expressed both in terms of mean grain

density and after transformation, as absolute values of ligand bound per mg tissue. Both suffer some interpretive problems. The non-linearity of the responsiveness of the emulsion at the lower and upper extremes makes the direct use of mean grain density as an expression of receptor density inevitably flawed. Because grain density over the naloxone controls, which are so close to background, are subtracted from grain density over sections with [^{3}H]ligand ± displacer, there is the potential for inaccuracy. This is why in transforming the data to absolute values, tissue mean grain densities were only corrected for naloxone control after both had been transformed to fmol ligand bound per mg tissue ensuring linearity in the relation between both quantities and accuracy. On the other hand, the use of the formula found to best describe the standard curve to transform mean grain densities to absolute values introduces an element of extrapolation. It was thought prudent given these potential pitfalls in the quantification of data from this sort of experiment to express the results both as mean grain density, the direct measure, and as ligand fmol bound per mg tissue, the extrapolated measure.

It is clear that whilst the proportions by which changes in receptor density are different in magnitude as expressed in the two ways, the direction of the change is the same (see MPN Results Section). I conclude therefore that the change in receptor density in MPN after chronic morphine treatment is a real one.

Because the quantitative receptor autoradiographic techniques and principles being applied here to an intact cell preparation were originally developed for use in isolated membrane preparations, there are conditions encountered here with brain sections which need to be taken into account when drawing conclusions from the data. These interpretive problems have been raised already (Motulsky et al, 1985; Dashwood, 1987). Because of the need to standardise results between laboratories there has been a trend to quantification of receptor binding in tissue sections in terms of fmol ligand bound per mg protein, an expression already commonly used in ligand binding studies in homogenates and similar to that used here. The first difficulty arises from the non-

random distribution of white matter in brain sections as compared with an equivalent homogenate, which will result in differential degrees of quenching of tritium depending upon the brain region under study. The way used to control for this in quantifying the present results was to use a brain homogenate-equivalent calibration factor on all mean grain densities to convert them to absolute values. For a review of technical procedures see Rogers, 1979 and Sumner, 1988.

5.4.3. Supraoptic nucleus

The results presented here reveal that there are κ-opioid receptors in the SON. Other studies have identified receptors of the κ-subtype in the SON (Mansour et al, 1986; Tempel and Zukin, 1987; Mansour et al, 1988) and dynorphin binding sites (Clark et al, 1986) so these results are not without precedent but the amount of binding measured in this study, 130.4 ± 25.6 in the control group and 95.5 ±16.0 fmol/mg tissue in the morphine-treated group cannot be compared to measurements in other studies as the only other quantitative autoradiographic studies in hypothalamus either probed SON but did not report a value for absolute binding of a κ-selective ligand (Clark et al, 1986) or did not specifically probe SON for opioid receptors (Tempel and Zukin, 1987). The technique used is a relatively low resolution technique and because the sections were apposed to the emulsion rather than coating the sections themselves with photographic emulsion, there is no way of identifying the elements within the SON to which silver grains were localised and it cannot be concluded therefore if the receptors labelled in this study are on the supraoptic neurones themselves, on the terminals of afferents in the SON or on the surrounding glial cells, the latter being the case in the posterior pituitary (Lightman et al, 1983a; Lightman et al, 1983b) where opioid receptors are also found (Simantov and Snyder, 1977). Other studies have also failed to address this question which might be best investigated by a higher resolution method which allows tissue counter staining to identify oxytocinergic neurones at the same time as measuring the distribution of silver grain over opioid receptors. One such method would involve the use of a specific monoclonal antibody to the opioid

receptor for light microscopic visualisation of opioid receptors with simultaneous counterstaining with an antibody against OT to enable resolution of the cellular localisation of the receptors. Opioid receptor distribution in rat brain sections has been investigated using such an antibody and although the data could not be quantified, the pattern was shown to be similar to that already demonstrated by receptor autoradiography (Hassan et al, 1989). Although antibodies specifically directed against the three subtypes of opioid receptor are not yet available, the antibody used in the above study could distinguish between μ/δ and κ-opioid receptors. Indeed, this was the first receptor autoradiographic study to report the existence of μ/δ-opioid receptors in the paraventricular and supraoptic nuclei (Sumner et al, 1990). This finding was not echoed in previous receptor autoradiography studies according to Hassan and coworkers because of the low numbers of μ/δ-opioid receptors in SON and paraventricular nucleus and the relatively low sensitivity of autoradiography compared with immunocytochemical techniques. This explanation puts into context the relatively moderate autoradiographic image produced over the SON in this study in comparison with much more dense patches in the caudate putamen for example (see Figure 5.3.1.3.). Of course, most importantly, even from this moderate image it was possible to discern a statistically significant change in optical density after chronic morphine treatment. This change in optical density, corresponding to specific opiate binding sites after chronic morphine treatment, was seen elsewhere in tissue sections evaluated subjectively by Dr. Sumner (personal communication) and was not a feature exclusively of the SON.

From the evidence from this study however, the location of κ-receptors in the SON can only be speculated upon. They might indeed be located on the OT neurones themselves which would be the most simple scenario in terms of explaining opiate effects on electrical activity of these neurones. Equally probable however is the location of opioid receptors presynaptically on the terminals of interneurones in the SON or upon the terminals of afferents to the SON. These two possibilities are not

mutually exclusive as the possibility of both presynaptically-located and postsynaptically-located opioid receptors has been raised elsewhere as a model helpful in explaining biphasic opiate effects (See Chapter 2). Opioid receptors of the three subtypes have been found in hypothalamus in a release study in hypothalamic slice. The release of endogenous opioid peptides was measured in the presence of receptor subtype-selective antagonists in the presence of tetrodotoxin to prevent axonal transmission. One conclusion of the study was that the three subtypes of opioid receptor are present in the hypothalamus and are located upon presynaptic terminals (Nikolaris et al, 1989). This might be the case in the SON. Finally opioid receptors might be located on the glia in the SON, the astrocytes. This would parallel what has been shown in the posterior pituitary where the pituicytes are carriers of functional opioid receptors (Lightman et al, 1983) which may partly mediate the inhibition of OT release upon stimulation by appropriate opioid ligands.

The plasticity of opioid receptors in the SON was demonstrated in another aspect of the present study (Sumner et al, 1990) when chronic morphine treatment was shown to markedly reduce the density of μ-opioid receptors in the SON. This finding demonstrates the responsiveness of this system to a chronic stimulus. But this is a differential responsiveness because as described here the density of κ-opioid receptors is unaffected by chronic morphine treatment, i.e. there is homologous down-regulation. From my own studies in which the possibility of cross-tolerance between the μ-agonist morphine, given chronically and the κ-agonist, U50,488H, given acutely, in terms of electrical activity in oxytocin (OT) neurones of the SON, I was unable to find evidence of changes in κ-receptor sensitivity whilst demonstrating a marked reduction in μ-receptor sensitivity (Leng et al, 1990; see also Chapter 2). So these earlier studies support the implication arising here that although cellular adaptation clearly occurs in response to chronic morphine exposure, it is restricted to the population of μ-receptors both in terms of receptor density and function.

5.4.4. Median preoptic nucleus

Because no attempt was made to identify the subtype(s) of opioid receptor in the MPN which is subject to adaptive changes after chronic opiate, the result obtained in SON must colour the interpretation of the down-regulation of opioid receptor clearly evident here. It seems likely that the subtype affected by chronic morphine is the μ-receptor but this could be investigated by the use of receptor subtype-selective displacers in a similar study to unambiguously identify the receptors affected.

I have already alluded to the questions of interpretation which must be considered when attempting to quantify such data as these. Where such a dramatic change in receptor density apparently occurs it is worth reflecting on the following points of controversy. Interpretation of radioligand binding studies often rests on the following assumptions, 1) all the receptors are accessible to all ligands; 2) the binding properties of the receptors do not change; 3) the number of receptors is constant 4) ligands are not altered 4) the concentration of modulators of receptor function do not change; and 6) the incubation reaches equilibrium' (from Motulsky et al, 1985). Although these assumptions are acceptable when applied to antagonist ligands, used in isolated membrane preparations, they do not necessarily hold true for agonist ligands used on intact cells. Taking the first point, it is possible that incubation with agonist ligands but not with antagonist ligands can cause a redistribution of cell surface receptors (clustering) preventing access to the full complement. Secondly, receptor conformation can be altered after interaction with ligand in a way which changes the subsequent interactions of the same receptor with fresh ligand, in terms of receptor binding studies this might mean a decreasing agonist binding affinity with time. Thirdly, receptor numbers might change during the course of an experiment carried out at physiological temperatures. The half-life of receptors ranges between hours and days and thus there may be a significant degree of receptor removal or insertion during a lengthy incubation protocol. The fifth point in Motulsky's list suggests that where agonists are present which modulate the availability of GTP, the interaction between

G-protein and receptor could be modified, consequently affecting the affinity of the receptor for its ligand. In the present study it might mean that the 100 x excess of displacers used to look at one particular receptor subtype could produce a change in intracellular GTP which would affect the affinity of the receptor under study for its ligand, thus reducing its apparent density. I would answer these points as they apply to this study by pointing out that although not fixed, the tissue used was first of all frozen on dry ice and was after sectioning and thaw mounting kept deep frozen for some time before being incubated at 0-4°C for the SON study and at room temperature for the MPN study. It was then exposed at 0-4°C for 9 weeks after which the autoradiographs were developed. So it seems unlikely that any of the above processes could occur to any significant degree between the time the brains were taken and the autoradiographs developed. Because such low temperatures were used throughout I believe that the density of receptors measured in this study reflects wholly changes in receptor manufacture/recycling and removal/phosphorylation (see later for discussion of these mechanisms) and the changes seen in MPN represent a physiological adaptive process after chronic morphine.

Opioid receptors have been reported in the hypothalamus (Mansour et al, 1986; Clark et al, 1986; Mansour et al, 1987; Tempel and Zukin, 1987; Mansour et al, 1988; Sharif and Hughes, 1989) but not specifically in MPN which we report for the first time (Sumner et al, 1990). The amount of [^{3}H]etorphine binding in MPN before and after chronic morphine treatment was 76.2 ± 9.8 and 17.5 ± 5.2 fmol/mg tissue. A direct comparison between the present values and those obtained in other quantitative autoradiographic studies cannot be made as MPN has not previously been probed for opioid receptors. However, another structure in the AV3V region, the subfornical organ (SFO) does contain opioid receptors, in order of relative density by receptor autoradiography, $\kappa \gg \mu > \delta$ (Mansour et al, 1987). A more recent study quantitatively analysed opioid receptor density in SFO (Sharif and Hughes, 1989) and found μ-opioid receptors in the SFO (195 amol/mm^2) and κ-opioid receptors in the SFO (2.5

amol/mm^2, 10μm sections), but again, MPN was not probed. SFO was probed using 4 anti-idiotypic antibodies for the δ-opioid receptor which was found qualitatively to be labelled very strongly, both at the level of the cell membranes (although not usually the synaptic membrane) and in cytoplasmic vesicles (Conrath and Cupo, 1989; see later discussion on receptor internalisation). This study extends the finding of opioid receptors in MPN by identifying circumstances under which the manufacture and/or the removal of these receptors are affected.

Is there a physiological correlate of these data? Are there circumstances under which endogenous opioids might be chronically elevated sufficiently to reproduce these changes? The answer is not immediately forthcoming. I had referred in a previous Chapter to changes in opioid tone possibly occurring during pregnancy in order to produce a sudden and dramatic rise in OT release to aid expulsion of the foetuses. Such a model would require a steady increase in opioid tone during gestation which would be withdrawn precipitously at the time of parturition. In fact opioid tone is high during parturition as revealed by naloxone, but OT plasma concentration is still elevated compared to virgin rats (Bicknell et al, 1988b). It is feasible that a prolonged increase in opioid tone in the MPN, similar to that earlier suggested to occur in the SON, could result in a down-regulation of receptor numbers and this could therefore be the physiological correlate of this finding. However, this hypothesis is not supported by recent evidence obtained from rats at various stages of pregnancy in which there was found to be no change in receptor density in the MPN at days 16, 20 and 21 compared to virgin rats (although SON μ-receptor density is decreased; personal communication, J.A. Russell/A. Douglas). So, receptor density changes in MPN do not easily fit with pregnancy as a physiological correlate. However, it could be that changes in receptor density are much more rapid than the pregnancy protocols are capable of detecting leaving a question-mark over the physiological significance of these observations (see later Discussion).

The presence of opioid receptors in the MPN and changes in MPN receptor density

with chronic morphine agree with earlier electrophysiological results (see Chapter 6) from which it was concluded that the development of tolerance and expression of dependence, manifested by the withdrawal response after naloxone, are impaired by the electrolytic ablation of the AV3V region, which includes MPN. Stimulation of the AV3V region excites OT neurones (Russell et al, 1988) and its ablation removes a tonic excitatory drive to OT cells, which are silenced (Russell et al, 1988). The AV3V region is essential in maintaining the osmotic responsiveness of OT neurones which are electrically excited by a fall in plasma osmolality and are slowed by a rise in plasma osmolality but its integrity is not a required for the normal progress of parturition and for milk-let down with suckling (Russell et al, 1988). So once again it is difficult to put the observed reduction in opioid receptor density into the context of the physiological changes that occur leading up to and during parturition. It remains to be shown however whether there is a measurable change in one subtype of opioid receptor which is masked by no change in the other subtypes of receptor, should they be present. It must also be considered that the method of introducing morphine to neurones of the MPN, adjacent to the 3V, might be somewhat crude in comparison with likely levels of endogenous opioids in the region even after their elevation by a physiological stimulus. Exogenous morphine concentration in the MPN might in fact equate with a more pharmacological than physiological level of agonist at the opioid receptors present in the MPN and might have induced a pharmacological response in mechanisms sensitive to endogenous opioids including those controlling receptor turnover. If this most pessimistic interpretation was true then the observed decrease in opioid receptor density in the MPN would model morphine tolerance rather than a physiological control mechanism.

Also intriguing is how receptor down-regulation after chronic morphine treatment and the observed hyperexcitation of these neurones after acute naloxone requiring a high degree of functional integrity, might be married with one encompassing hypothesis. This question has been addressed and based on observations in several

cell types, excluding SON OT neurones, it has been suggested that tolerance and dependence might be quite separate phenomena (Wuster and Costa, 1984; Wuster et al, 1985; Christie et al, 1987). Perhaps the two are in fact separate phenomena: the decline in receptor density might reflect a mechanism contributing just to tolerance but not to dependence. Possibly more important for the expression of withdrawal excitation is the functional state of the receptor or of post-receptor mechanisms which might be temporarily inactivated by chronic opiate but rapidly reinstated following naloxone. The system needs to be capable of not only behaving normally, but supernormally after naloxone, which suggests that the receptor-post-receptor organisation is not permanently dismantled but is merely temporarily interfered with. Transient opioid μ-receptor desensitisation in locus coeruleus neurones has been described after 5min application of met-enkephalin (Harris and Williams, 1990) which is homologous because the α_2-adrenoceptor is not substantially affected. The desensitisation lasts for 15-20min and must therefore involve a modification of the receptor which is rapid and probably reversible (see below for a discussion of possible mechanisms).

There is evidence from a variety of receptors to explain reversible or irreversible receptor inactivation, although evidence from studies of the opioid receptor specifically are sparse (Law et al, 1984; Law et al, 1985; Hassan et al, 1989; Conrath and Cupo, 1989).

5.4.5. Regulation of receptor density by internalisation

Receptors can undergo internalisation by endocytosis within 'coated pits' and can then either be broken down within lysosmes or preserved (Dickson, 1985). Endocytosis can be substantial: cells may internalise the equivalent of up to 150% of the total cell surface per hour (Raffa, 1985). In addition, it seems that some coated pits only bud off from the cytoplasmic membrane after the creation of a ligand-receptor complex although others do not require the binding of a receptor-selective ligand to promote this process. Once internalised, contents of these coated vesicles

can be enzymatically degraded, secreted after modification or reinserted into the membrane unchanged (Raffa, 1985). It is easy to speculate on the role of such a system in opioid receptor down-regulation and desensitisation. Opioid receptors labelled by an anti-idiotypic antibody directed against the μ/δ-opioid receptors appear to be located not only at the cell membrane of various neurones throughout the rat brain and of NG108-15 hybrid cells, but also within the cytoplasm, although this can only be confirmed convincingly by electron-microscopical analysis (Hassan et al, 1989). δ-opioid receptor binding measured using an anti-idiotypic antibody in rat brain was localised not only to cell membrane but aso intracellularly within vesicles as confirmed by electron microscopical examination (Conrath and Cupo, 1989). δ-opioid receptor down-regulation in NG 108-15 hybrid cells after chronic DADLE has been reported to be accompanied by an increase in the lysosomal compartment of ligand-receptor complex, where degradation takes place (Law et al, 1984). It was concluded that because opioid receptor desensitisation requires magnesium and sodium which are needed for activation of Gi and AC respectively, that the opioid receptor needs to be coupled to Gi for desensitisation to occur. If internalisation is a significant means of opioid receptor desensitisation then it might be expected that the opioid receptor would be internalised together with the coupled Gi protein. However, it has been shown that the amount of Gi in the membrane does not change after chronic DADLE and significant opioid receptor internalisation (Law et al, 1985). This is consistent with homologous desensitisation having occurred in that if Gi was significantly depleted in the plasma membrane, heterologous desensitisation would be expected because of the general depletion of available Gi for transduction via other receptors (see Chapter 3). As post-receptor mechanisms are not shown to be causally involved in receptor internalisation by the above study, and cell-surface receptor clustering occurs both with agonists (including morphine) and with naloxone and cannot therefore be a candidate pre-internalisation marker, the authors of the above study speculate on the possible role of covalent modification of the opioid receptor upon

binding of the agonist (ie. phosphorylation) which might deactivate the receptor and lead to its internalisation (see 5.4.6. for further discussion of receptor phosphorylation). A recent report raises an interesting corollary to the above findings, which is the observation that the opioid antagonist, naloxone causes an accumulation of opioid receptors in the lysosmal fraction of rat brain, albeit a relatively small one, suggesting that opioid receptor up-regulation might operate by interfering with receptor internalisation and processing (Simantov et al, 1989).

Recycling of receptors has been reported to occur in as little as 30min (Raffa, 1985) although there is no figure specifically for opioid receptors. So, the decrease in density of opioid receptors observed here might be accounted for, should these processes operate significantly for the opioid receptor, by the reversible internalisation of receptors which might be preserved internally for later recruitment for example after exposure of the cells to naloxone. This could provide the basis of a unitary explanation based primarily upon receptor density for tolerance followed by supernormal activity after naloxone - dependence in a cell-surface receptor-depleted preparation. A remaining difficulty however is whether receptors can be recycled into the membrane sufficiently rapidly to account for the very short latency in the onset of the withdrawal response seen in electrophysiological studies of these neurones (approx. 30sec; see Chapter 1). This might be answered if internalised receptors with their ligand although internalised were accessible to naloxone. Some ligands are indeed internalised but this scenario seems improbable. Alternatively rapidly reversible deactivation by another means which renders the receptor less avid for its selective ligands might make the ligands used in this study blind to cell-surface receptors which nevertheless are quickly reinstated after a hypothetical conformational change induced by naloxone. Perhaps receptor phosphorylation operates in this way as it would not necessarily entail removing the receptor from the membrane as part of its deactivation, but evidence to support this idea is lacking. An alternative interpretation is that although the down-regulation of receptors could contribute to

tolerance possibly by internalisation, a separate mechanism(s) probably accounts for dependence (Wuster et al, 1984; Wuster et al, 1985; Christie et al, 1987). It is interesting that after internalisation of the phosphorylated β-adrenergic receptor the phosphate residue is removed, presumably by a phosphatase present in the vesicle (Levitzki, 1986). Thus the functional receptor could be reinserted into the plasma membrane. The period of receptor internalisation would vary under conditions of tachyphylaxis (a short internalisation) or tolerance (a longer internalisation). These experiments have not been performed for opioid receptors so it is not clear whether these results can be reliably extrapolated.

5.4.6. Regulation of opioid receptor density by phosphorylation

Many modifications can be made to receptor proteins before their transport to the membrane, possibly to provide resilience to enzymatic attack. However, various receptors are phosphorylated on serine, threonine and tyrosine residues after ligand binding (Hanover and Dickson, 1985). For example, insulin induces rapid phosphorylation of tyrosine residues at its receptor triggering a cascade of such phosphorylations and ultimately reducing tyrosine kinase activity.

Huganir and Greengard list three classes of receptor, all of which can be phosphorylated as a means of regulation: chemically-gated ion channels, those linked to guanine nucleotide binding proteins and receptors for growth factors (Huganir and Greengard, 1987). An example of the second class of receptor, to which opioid receptors belong, is the β-adrenergic receptor. Phosphorylation of the β-adrenergic receptor results in desensitisation. The β-adrenergic receptor undergoes two forms of desensitisation: homologous and heterologous. That is, desensitisation brought about by the binding of an appropriate ligand to the β-adrenergic receptor, or desensitisation of unrelated receptors after binding of the same ligand again to the β-adrenergic receptor. Protein phosphorylation is involved in the development of both forms of desensitisation. Phosphorylation can be catalysed by cAMP-dependent protein kinase or protein kinase C, activated by phorbol esters in heterologous desensitisation. In the

case of homologous desensitisation, a novel protein kinase is involved (β-adrenergic receptor protein kinase, β-ARK), and the degree of phosphorylation correlates well with the degree of desensitisation in frog erythrocytes. This type of phosphorylation occurs only when the ligand is bound to the receptor and is therefore, substrate-activated. Binding of the agonist (but not the antagonist) seems to make the receptor a better substrate for phosphorylation. β-ARK promotes functional inactivation of the receptor and internalisation, whereas protein kinase C marks the receptor for internalisation at a different site, without functionally inactivating it. Thus it has been suggested that phosphorylation is a necessary first step in the internalisation of the receptor (Levitzki, 1986).

Tolerance and dependence in the OT system have not been dissociated by the protocols described in thid Thesis, where they seem to develop hand in hand. In systems where tolerance and dependence have been shown to progress at different rates or to be independent of each other, it has been necessary to explore hypotheses other than the unitary hypothesis of tolerance and dependence. The present studies do not provide evidence to exclude either a unitary hypothesis or other proposed hypotheses in the OT cells of the SON and I leave this question for future clarification.

Decline in receptor density could be one of many adaptive mechanisms which contribute to tolerance and dependence and it is certainly naive to expect to derive a convincing hypothesis based on changes in receptor density alone. Indeed, in some neuronal systems, there is no change or an increase in the density of opioid receptors after chronic opiate, suggesting that it is not a universally active adaptive mechanism under conditions of chronic opiate exposure (Geary and Wooten, 1985; Brady et al, 1989).

In conclusion, localisation of κ-opioid receptors in the SON gives biochemical validity to the electrophysiological effects of the κ-selective agonist U50,488H and other κ-receptor agonists in vitro and in vivo (Pumford et al, 1987; Russell et al, 1989b; Inenaga et al, 1990; Leng et al, 1990). That the density of these receptors is

not influenced by chronic morphine treatment corroborates electrophysiological findings which suggest no cross-tolerance occurs between the chronically-inactivated μ-opioid receptor and the acutely activated κ-opioid receptor (see Chapter 2). The localisation of opioid receptors in the MPN is a novel finding, as is the change in opioid receptor density evoked by chronic i.c.v. morphine-treatment and is supportive of the electrophysiological study (see Chapter 6) in which it was found that at least part of the adaptation that constitutes tolerance/dependence in SON OT neurones occurs in the AV3V region, of which the MPN is a part (see Chapter 6). Clearly in vivo, morphine could act to inhibit SON OT neurones partly by an action on the input from the MPN. These data then are concordant with other studies reported in this Thesis of the OT neurosecretory system in the SON, as it is regulated by endogenous and exogenous opioids. Further work might concentrate on identification of the peptidergic nature of neurones in the SON carrying subtypes of opioid receptor and could attempt to clarify the likely physiological relevance of these findings to date.

CHAPTER 6

MECHANISMS MEDIATING DEPENDENCE UPON MORPHINE IN OXYTOCIN NEUROSECRETORY NEURONES: WHERE ARE THEY LOCATED?

6.1. INTRODUCTION

Oxytocin (OT) is released together with vasopressin (AVP) from the posterior pituitary in the rat in response to osmotic stimuli, such as intraperitoneal injection of hypertonic saline or chronic dehydration (Brimble et al, 1978) and this is the result of an increase in the rate of firing of oxytocin neurones (Poulain et al, 1977).

Direct osmosensitivity of oxytocin neurones has been inferred from in vitro studies (Mason, 1980) and confirmed by in vivo experiments (Leng et al, 1989a) but complementary to their innate osmosensitivity, these neurones also receive afferents from regions elsewhere in the brain, which may themselves be osmosensitive sites.

The region anterior and ventral to the third ventricle (AV3V region) includes the organum vasculosum of the lamina terminalis (OVLT), the median preoptic nucleus (MPN) and connections between these and the subfornical organ (SFO) (Brody and Johnson, 1980). Lesions in the AV3V region upset the maintenence of water and sodium balance (Johnson and Buggy, 1978). AVP and OT release associated with water deprivation are also inhibited by these lesions (Bealer et al, 1983; Russell et al, 1984) and terminal degeneration is observed in the paraventricular and supraoptic nuclei (PVN and SON respectively; Carithers et al, 1981), where both the SFO and the OVLT are known to project to (Miselis, 1981; Renaud et al, 1983).

Stimulation of the AV3V region, most effectively in and around the MPN causes the release of OT in both lactating and non-lactating rats (Russell et al, 1988). Honda and coworkers showed that by increasing osmolality discretely in the OVLT by microinjection, the firing rate of paraventricular neurones was increased (Honda et al, 1987). Although the milk ejection reflex remains intact after electrolytic ablation of the AV3V region and parturition progresses normally after acute AV3V lesion (Russell et al, 1988), the osmotically-stimulated increase in plasma OT concentration is no longer seen (Blackburn et al, 1987). So, the AV3V region selectively influences the osmoresponsiveness of the magnocellular OT neurones without affecting their

ability to respond to the suckling stimulus with a coordinated increase in firing activity resulting in the bolus release of OT and milk ejection or to the passage of a foetus through the vagina with an increase in oxytocin release.

Stimulation of OT neurones by suckling, angiotensin II and cholinomimetics is sensitive to the acute inhibitory effects of opiate receptor agonists in a naloxone reversible way (Haldar and Sawyer, 1978; Clarke et al, 1979; Haldar et al, 1982; Russell and Spears, 1984). Endogenous opioid peptides may also exert a tonic inhibitory influence on OT neurones (Bicknell et al, 1984).

Chronic exposure of the OT system to morphine has revealed the development of tolerance and dependence to these inhibitory effects (Russell, 1984; see Chapter 1)). The relative influence of neural inputs to the OT system in the normal state and in the opiate tolerant/dependent state could lead to a better understanding of the mechanisms involved in opiate tolerance and dependence. In addition, if tolerance and dependence develop to exogenous opioids then the possibility must exist that the same might occur to endogenous opioids under certain physiological conditions. Thus the influence of neural inputs in the tolerant state would have a role in the coordination of the system tolerant to its own endogenous opioids (Lincoln and Russell, 1985)

In this study the contribution of the AV3V input to the expression of tolerance and dependence in the OT system is examined, revealed by precipitated withdrawal with the opiate antagonist naloxone, in terms of plasma OT concentration measured by radioimmunoassay. Specifically, it is hypothesised that if morphine dependence develops only in the AV3V input to OT neurones, then the AV3V lesion should prevent withdrawal excitation. From the results, possible mechanisms by which naloxone affects OT neuronal activity and therefore OT release, in i.c.v. vehicle-infused and i.c.v. morphine-infused rats, with and without acute electrolytic ablation of the AV3V region, are considered.

6.2. METHODS

6.2.1. Animals

Virgin female Sprague-Dawley rats (255.8 ± 21.6g, i.c.v. vehicle-infused group, n=12; 264.9 ± 6.5g, i.c.v. morphine-infused group, n=12) were used in this study. They were housed singly with access to food (standard breeder diet) and water ad libitum in a 12hour light dark cycle (13h/11h light/dark) at an ambient temperature of 21-23°C.

6.2.2. Intracerebroventricular (i.c.v.) infusion procedure

Rats to be included in the chronic morphine treatment group were taken 5 days before the experiment and were anaesthetised with ether. Using a stereotactic apparatus and atlas of the rat brain (Konig and Klippel, 1963), a cannula was introduced into a lateral cerebral ventricle (3mm posterior, 2mm left lateral to bregma, 4.5mm below the surface of the skull) and an infusion of morphine sulphate BP (Pharmacy, Royal Infirmary, Edinburgh) at consecutive concentrations of 10, 20 and 50 mg/ml was set up, driven by a subcutaneously implanted osmotic minipump (Alzet 2001, Alza Corp.). Control rats were prepared identically but received an i.c.v. infusion of sterile pyrogen-free water.

6.2.3. Electrolytic lesioning / sham-lesioning procedure

Rats were anaesthetised with urethane (0.5ml/100g of 25% ethyl carbamate i.p.) and the left femoral artery and vein cannulated for blood sampling and injections respectively. Body temperature was regulated to 37°C using thermostatically controlled small animal heating blankets.

Half of each group received an electrolytic lesion in the AV3V area (0.3mm posterior to bregma and 8.3mm below the surface of the skull with the rat skull level between bregma and lambda). A lesioning current, 3mA D.C. was passed for 15 seconds from a Stoelting lesion maker (58041, Chicago, IL, U.S.A.; rectal cathode) using a lesioning electrode (Nichrome; Goodfellow metals, Cambridge; 26 SWG,

0.5mm diameter insulated to 0.5mm of the tip with Insl-X; Insl-X Products Corp., Yonkers, NY, U.S.A.). The remainder received a sham lesion, which involved the introduction of the lesioning electrode dorsal to the AV3V without the passage of a lesioning current (0.3mm posterior to bregma and 5.3mm vertically beneath the skull surface; no current). Pairs of lesioned and sham-lesioned rats were prepared together.

6.2.4. Blood sampling protocol

The blood sampling schedule was as follows,

Blood sample number:

1 Just prior to lesion/sham lesion, rat in frame

Electrolytic lesion / sham-lesion procedure

2 1 minute after making lesion/sham-lesion

3 30 minutes after making lesion/sham-lesion

4 60 minutes after making lesion/sham-lesion

Naloxone (5mg/kg) i.v. 65 minutes after lesion/sham-lesion

5 6 minutes after naloxone

6 20 minutes after naloxone

7 40 minutes after naloxone

8 60 minutes after naloxone

Naloxone (5mg/kg, 10mg/ml solution in 0.9% saline; either a gift from Endo Labs. or purchased from the Sigma Chemical Co.) was given by intravenous bolus injection 65 minutes after the lesion or sham procedure. Blood samples were 400μl and were replaced with heparinised blood from a 400g donor rat. They were kept on ice until the plasma was separated by centrifugation at 0°C and stored frozen at -20°C before radioimmunoassay for OT by a modified Higuchi method (Higuchi et al, 1985). Details of the radioimmunoassay procedure are contained in the Methods Section of Chapter 4 and it was performed by Chris Chapman, AFRC I.A.P.G.R., Cambridge.

After collection of the last blood sample, animals were killed, the brains removed and fixed in 10% v/v formaldehyde for later examination. The site of the electrolytic

lesion and the placement of the i.c.v. cannula were confirmed by sectioning the brains on a freezing microtome (50μm coronal sections), staining sections with cresyl violet followed by serial examination under a dissecting microscope and recording of the spread of the lesion on semi-diagrammatic maps of rat brain derived from the atlas of Konig and Klippel (see Figures 6.4.1. and 6.4.2.a and b for a record of the extent of a typical AV3V lesion and for a composite summary of all lesions made in the control (a) and the i.c.v. chronic morphine-treated (b) groups). Accurately-placed lesions were those which crossed the mid-line and destroyed tissue ventral to the anterior commisure without involving the magnocellular nuclei directly; data from rats with mis-placed lesions were not included in subsequent analysis.

6.3.5. Statistical analysis of results

For comparison between groups a non-parametric analysis of variance was initially performed to locate any differences (Kruskal-Wallis) and once located, further comparisons were made with the Wilcoxon test for independent samples. For comparison of paired data (i.e. changes in OT concentration with time in any one group) the Wilcoxon signed rank test and Student's paired one-tailed or two-tailed t-test were used where appropriate. Student's t-test was used because of the inadequate sample size at some time points (where n=5), such that a non-parametric test could not detect a difference between samples. However, the normality of the data was tested with the Kolmogorov-Smirnov one sample test in which the control data (pre-lesion blood sample) was tested against a theoretical normal distribution. In all groups the data were found to be distributed normally (see Results Section) and parametric tests were then applied. The one-tailed t-test was the test of choice when a rise in plasma OT concentration was predicted from earlier published experimental evidence and was therefore expected. In all cases where this test was applied, plasma oxytocin concentration did indeed rise (see Results Section). All statistical analyses are presented in Tables 6.3.1., 6.3.3. and 6.3.4. of the Results Section of this Chapter.

6.3. RESULTS

Immediately prior to the AV3V lesion/sham lesion procedure plasma OT concentration did not differ between treatment groups. The plasma samples taken immediately after and at 30min after the lesion were likewise similar between groups. However, 60 minutes after the lesion the chronic i.c.v. vehicle-treated/lesioned (V/L) group had a lower plasma OT concentration than the chronic i.c.v. vehicle-treated/sham-lesioned (V/S) group ($P<0.05$, Wilcoxon, see Table 6.3.4. and Figure 6.3.2.) but neither was significantly different from its own pre-lesion control value (Student's paired, one-tailed t-test). This trend is mirrored in the chronic i.c.v. morphine-treated/sham-lesioned (M/S) and chronic i.c.v. morphine-treated/lesioned (M/L) groups. The pre-naloxone value for M/S was significantly higher than its own pre-lesion control value (Student's paired, one-tailed t-test, $P<0.05$). The pre-naloxone value for the M/L group was similar to its own pre-lesion control value. Thus whereas plasma OT concentration remained stable after a lesion, it tended to rise over the 60 min following the sham-lesion in both the i.c.v. vehicle- and i.c.v. morphine-treated groups.

Naloxone precipitated a massive rise in plasma OT concentration in the M/S group to a peak of 1839 ± 809 pg/ml, a greater than 80-fold increase on the pre-lesion control value ($P<0.05$, Student's paired, one-tailed t-test). This response compares well with that already observed in previous studies (Bicknell et al, 1988a) in non-operated morphine dependent rats.

All groups demonstrated a rise in plasma OT concentration after naloxone.

The M/L group showed an attenuated response compared to its sham-lesioned control (M/S), reaching a peak value of only 326.3 ± 65 pg/ml OT. However, although the absolute rise on plasma OT was much greater in the M/S group, the percentage increase in the M/L group after naloxone, compared with the M/S group was not significantly different (Wilcoxon test for independent samples).

The V/S group exhibited a significant increase in plasma OT concentration with naloxone compared with the pre-naloxone value: 67.3 ± 31.2 to 251.1 ± 66.5 pg/ml respectively, (ca. 4-fold increase; $P<0.005$, Student's paired, one-tailed t-test).

The V/L group was the least responsive of all to naloxone treatment and showed only a modest rise in plasma OT concentration from 15.2 ± 7.1 to 47.2 ± 12.4 pg/ml (ca. 3-fold increase; $P<0.05$, Student's paired, one-tailed t-test).

Plasma OT concentration was still elevated for 3 of the 4 treatment groups 60 min after naloxone (see Table 6.3.3.) but in all 4 groups for 40 min after naloxone (Wilcoxon signed rank test and Student's paired, one-tailed t-test). Only the V/L group did not vary greatly throughout the protocol in terms of plasma OT concentration. Comparisons between groups are made using the Kruskal-Wallis test for analysis of variance and the Wilcoxon test for independent samples. See Table 6.3.1. and Figure 6.3.2. for summaries of these data.

Comparison was made between groups at each time point using the Kruskal-Wallis test. Only at and after time point 4 (60min after the lesion/sham-lesion procedure) were there any statistically significant differences in plasma OT concentration. At time points 4, 5, 6, 7 and 8, P was <0.05, $=0.0005$, <0.005, <0.01 and <0.05, respectively. Differences between groups were further tested using the Wilcoxon test for independent samples where the differences occurred and the results are contained in Table 6.3.4.. The rank order of the group mean values for plasma OT concentrations after naloxone (time point 5) were M/S > V/S =M/L > V/L.

All rats included in our study had lesions which crossed the mid-line and fell beneath the anterior commisure and above the optic chiasm, damaging the ventral portion of the MPN and the OVLT but leaving both the SON and PVN untouched. The sham-lesioned rats had tissue damage above the anterior commisure, corresponding to the track of the lesioning electrode (see Figures 6.4.1. and 6.4.2.). The intra-assay coefficient of variation for the radioimmunoassay for oxytocin was 8.3%.

Table 6.3.1. Effect of AV3V lesion and chronic i.c.v. morphine infusion on plasma oxytocin concentration

	Treatment group			
Sample number	Vehicle/ sham (n=6)	Vehicle/ lesion (n=6)	Morphine/ sham (n=6)	Morphine/ lesion (n=6)
	Plasma oxytocin concentration (pg/ml)			
1 (0)	24.5 ± 7.1	13.6 ± 4.4	22.8 ± 3.7	18.5 ± 4.1
Lesion				
2 (1)	24.5 ± 7.9	19.8 ± 4.8	20.7 ± 3.5	33.3 ± 8.3
3 (30)	42.3 ± 13.3	17.3 ± 5.02	31.6 ± 9.1	34.3 ± 10.8
4 (60) (* KW)	67.3 ± 31.2	15.2 ± 7.1	65.3 ± 15.2	24.3 ± 7.8
Naloxone				
5 (71) (* KW)	251.1 ±66.5	47.2 ± 12.4	1839.0 ± 809.0	326.3 ± 65.0
6 (85) (* KW)	358.9 ± 112.1	54.6 ± 13.8	678.5 ± 136.4	315.3 ± 74.0
7 (105) (* KW)	283.3 ± 61.1	66.5 ± 17.9	554.2 ± 128.4	249.5 ± 69.3
8 (125) (* KW)	417.3 ± 19.0	55.4 ± 18.5	346.9 ± 79.8	195.5 ± 59.2

Table 6.3.1. Legend:

The number in parentheses together with the sample number denotes time in minutes at which blood samples were taken. (*KW) denotes a statistically significant difference according to non-parametric analysis of variance by the Kruskal-Wallis test

at that time point. Plasma OT values are mean ± SE. Naloxone was injected i.v. at 65 min (5mg/kg) and the next blood sample followed 6 min later.

Figure 6.3.2. (overleaf):

a) The groups V/S, V/L, M/S and M/L are as defined in the text. The lesion/sham-lesion procedure was performed after blood sample 1, as indicated by the asterisk. Naloxone was injected i.v. between blood samples 4 and 5 as indicated by the bold arrow. For a full account of where statistically significant differences were detected, see Tables 6.3.3. and 6.3.4..

b) This histogram was constructed using data from the first four blood samples only on an expanded y-axis in order to make the differences between groups at these relatively low plasma oxytocin concentrations more apparent. The asterisk denotes when the lesion/sham-lesion procedure was carried out and the bold arrow where naloxone was injected.

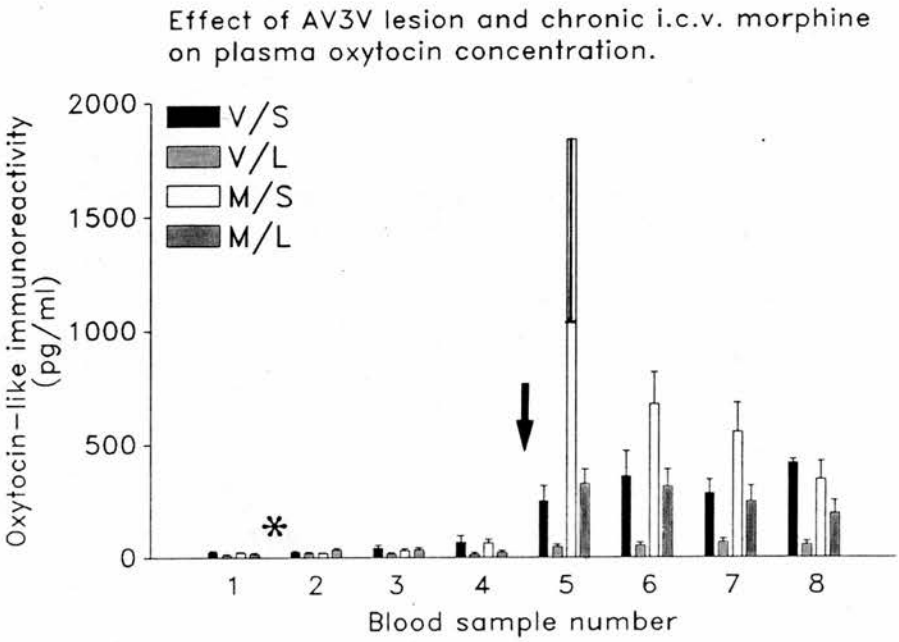


Figure 6.3.2.a

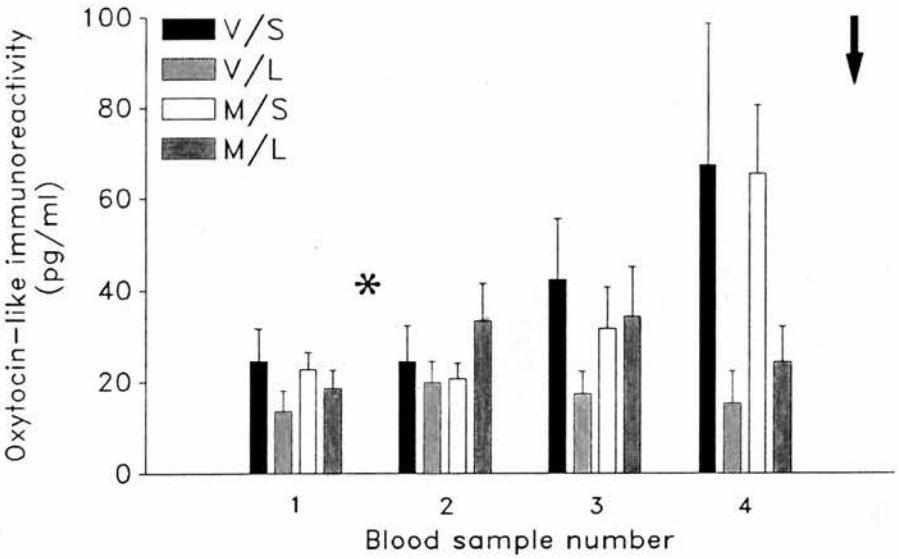


Figure 6.3.2.b

Table 6.3.3. Statistical analysis of the change in plasma oxytocin concentration with time after AV3V-lesion/sham-lesion procedure in morphine-naive and chronic i.c.v. morphine-infused rats.

Group code	Time point (min after naloxone)	vs. pre-naloxone value (4); Wilcoxon signed rank test, P<	vs. pre-naloxone value (4); paired, one-tailed t-test, P<	Group size
V/S	5 (6)	0.05	0.005	6
	6(20)	0.05	0.01	6
	7(40)	-----	0.01	5
	8(60)	-----	0.05	5
V/L	5(6)	-----	0.05	5
	6(20)	-----	0.05	5
	7(40)	-----	0.05	5
	8(60)	-----	>0.05 n.s.	5
M/S	5(6)	0.05	0.05	6
	6(20)	0.05	0.005	6
	7(40)	-----	0.01	5
	8(60)	-----	0.01	5
M/L	5(6)	0.05	0.005	6
	6(20)	0.05	0.005	6
	7(40)	0.05	0.01	6
	8(60)	0.05	0.05	6

Table 6.3.3. Legend

The group codes represent the following groups, V/S, the chronic i.c.v. vehicle-treated/sham-lesioned group; V/L, the chronic i.c.v. vehicle-treated/AV3V-lesioned group; M/S, the chronic i.c.v. morphine-treated/sham-lesioned group and M/L, the chronic i.c.v. morphine-treated/AV3V-lesioned group. The time points (5, 6, 7 and 8) each correspond to a serial blood sample taken at times given in parentheses. Values for P are absent (-----) in the Wilcoxon signed rank test column when statistical significance could not be achieved because the number of pairs was less than 6. These comparisons were then made using Student's paired, one-tailed t-test. The normality of the data was tested using the Kolmogorov-Smirnov one sample test. The plasma OT concentration measured in the pre-lesion control blood sample was compared with a normal distribution and for all 4 groups tested, the level of significance achieved was 1.00, satisfying the requirement of parametric tests such as Student's t-test for the data to be normally distributed. n.s. denotes that statistical significance was not achieved.

Table 6.3.4. Statistical analysis of plasma oxytocin concentrations before and after the AV3V-lesion/sham-lesion procedure in morphine-naive and chronic i.c.v. morphine-infused rats: comparisons between groups.

		Group code		
Group code	Time point	V/L	M/S	M/L
V/S	4	0.05	>0.05 n.s.	>0.05 n.s.
	5	=0.005	0.05	>0.05 n.s.
	6	0.01	>0.05 n.s.	>0.05 n.s.
	7	0.05	>0.05 n.s.	>0.05 n.s.
	8	0.05	>0.05 n.s.	>0.05 n.s.
V/L	4		>0.05 n.s.	>0.05 n.s.
	5		=0.005	=0.005
	6		=0.005	=0.005
	7		0.01	>0.05 n.s.
	8		0.05	0.05
M/S	4			>0.05 n.s.
	5			0.05
	6			>0.05 n.s.
	7			0.05
	8			>0.05 n.s.

Table 6.3.4. Legend

Group codes represent the following groups, V/S, the chronic i.c.v. vehicle-treated/sham-lesioned group; V/L, the chronic i.c.v. vehicle-treated/AV3V-lesioned group; M/S, the chronic i.c.v. morphine-treated/sham-lesioned group and M/L, the chronic i.c.v. morphine-treated/AV3V-lesioned group. The time points each correspond to a serial blood sample. Blank spaces occur where P values are expressed elsewhere in the table. Comparison between groups were made with the Wilcoxon test for independent samples and unless the chosen level of significance (P<=0.05) is not achieved, values are P< the stated value in the table. Where the value of P exceeds 0.05, this is expressed. n.s. denotes that statistical significance was not achieved. Group sizes were 5 or 6 rats throughout.

6.4. DISCUSSION

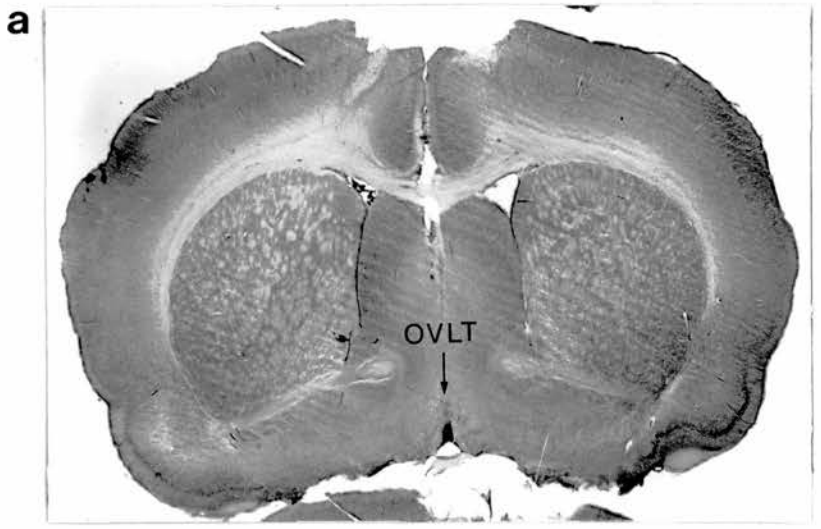
Photomicrographs of serial tissue sections from one rat (Figure 6.4.1.a-f) show the extent of a typical AV3V lesion. Figure 6.4.2.a and b are diagramatic composites of lesions in individual rats in the i.c.v. chronic vehicle-treated group (a) and the i.c.v. chronic morphine-treated group, showing the area common to all lesions. It is clear that neither the SON nor the PVN are affected directly by the AV3V lesion and any impairment of OT secretion in the lesioned groups cannot therefore be accounted for through destruction of the OT neurones or their axons. As well as the histological evidence, it would be expected that plasma OT would be reduced shortly after the lesion if the OT neurones themselves were substantially damaged and presumably OT release could not be stimulated by naloxone, but neither instance arose.

The first observation of note is that before the lesion procedure, plasma OT concentration was the same in all 4 groups (Kruskal-Wallis). Evidently, the chronic i.c.v. morphine-treated rats had become tolerant to morphine in terms of OT secretion since acute morphine inhibits OT secretion (Russell and Spears, 1984; Evans and Olley, 1988; Grell et al, 1988; Russell et al, 1989a). This agrees with an earlier study (Bicknell et al, 1988a) and is explained by tolerance to chronic i.c.v. morphine in terms of the electrical activity of OT neurones in the SON and PVN (see Chapter 1 and Bicknell et al, 1987a; Leng et al, 1989b).

During the post-lesion control period (time points 2-4) blood sampling revealed an interesting trend. OT output remained stable with time after the lesion procedure in the lesioned groups (V/L and M/L) but the sham-lesioned groups (V/S and M/S) showed an upward trend in plasma OT concentration (see Table 6.3.1.). Although this became maximal after 60 min in both the sham-lesioned groups, it is statistically significant only in the M/S group ($P<0.05$, Student's paired, two-tailed t-test). The pre-naloxone plasma OT concentration in the V/S and V/L groups (at time point 4) were significantly different from each other ($P<0.05$, Wilcoxon test for independent

samples) but no other comparisons achieved statistical significance. The stability of plasma OT in the lesioned groups suggests that acute ablation of the AV3V region removes a regulatory mechanism located in or projecting via the AV3V region which provides a positive drive to OT secretion which is possibly tonically active under the conditions of the experiment. However, this positive drive to the OT-secreting neurones in the SON is not essential for the secretion of what appear to be basal levels of OT by the remainder of the OT neurosecretory system (see Table 6.3.1.and later Discussion).

The reason for the gradual rise in plasma OT may be related to the consecutive blood samples taken during the post-lesion period, but these were always replaced with the same volume of blood from a donor rat, given immediately, to avoid this potential problem. In addition, there may have been slight blood loss with surgery which would have the same effect (Poulain et al, 1977). Again, care was always taken to minimise this problem. Both however, could result in the activation of OT neurones through the AV3V region, possibly via increased circulating AII, with the observed gradual rise in plasma OT concentration (Nikolaidis et al, 1983; Ferguson and Renaud, 1986; Gambino and Felix, 1986; Leng et al, 1989a). Fortunately, the pre-naloxone concentration of OT in these two groups was not sufficiently raised as to obscure the post-naloxone response.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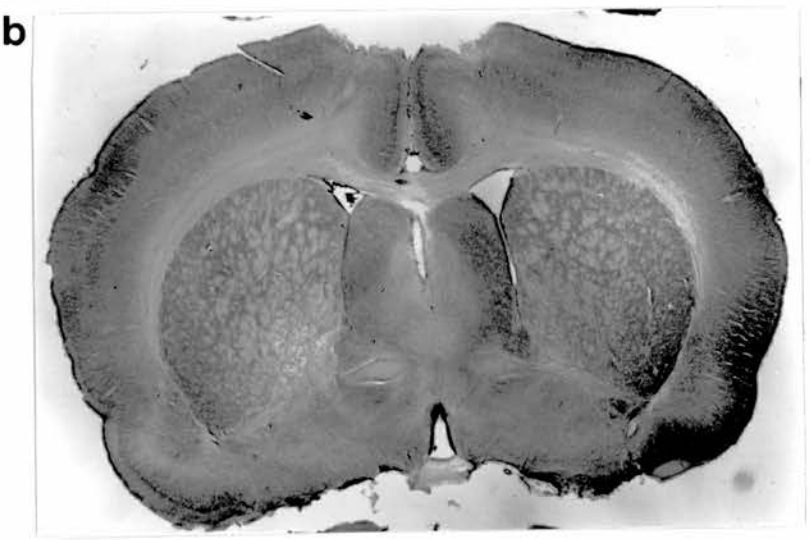
OVLT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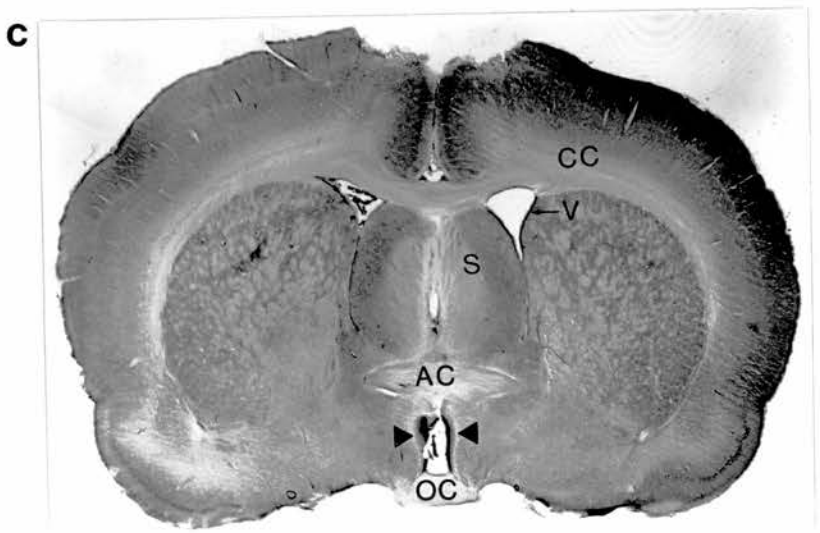
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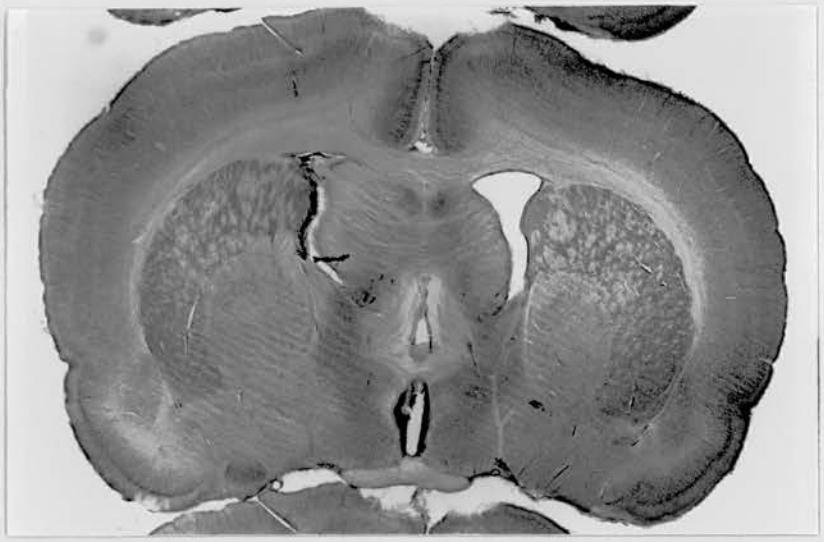

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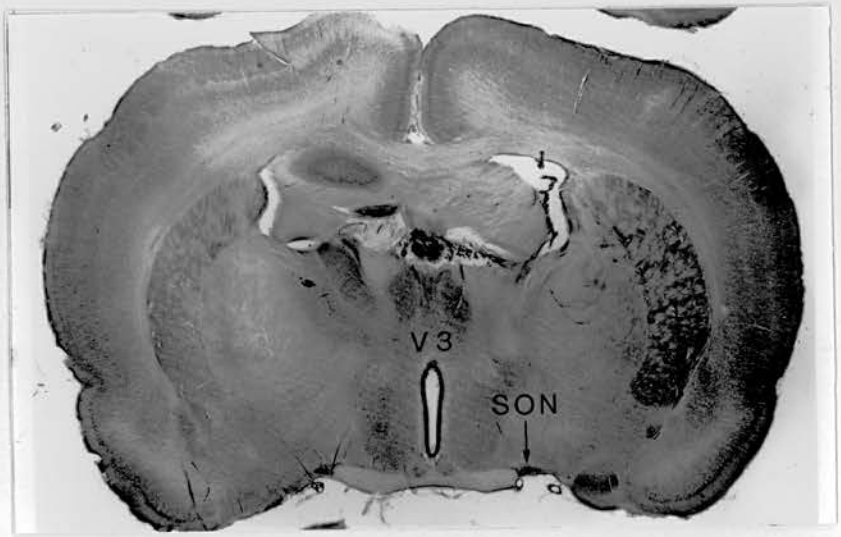

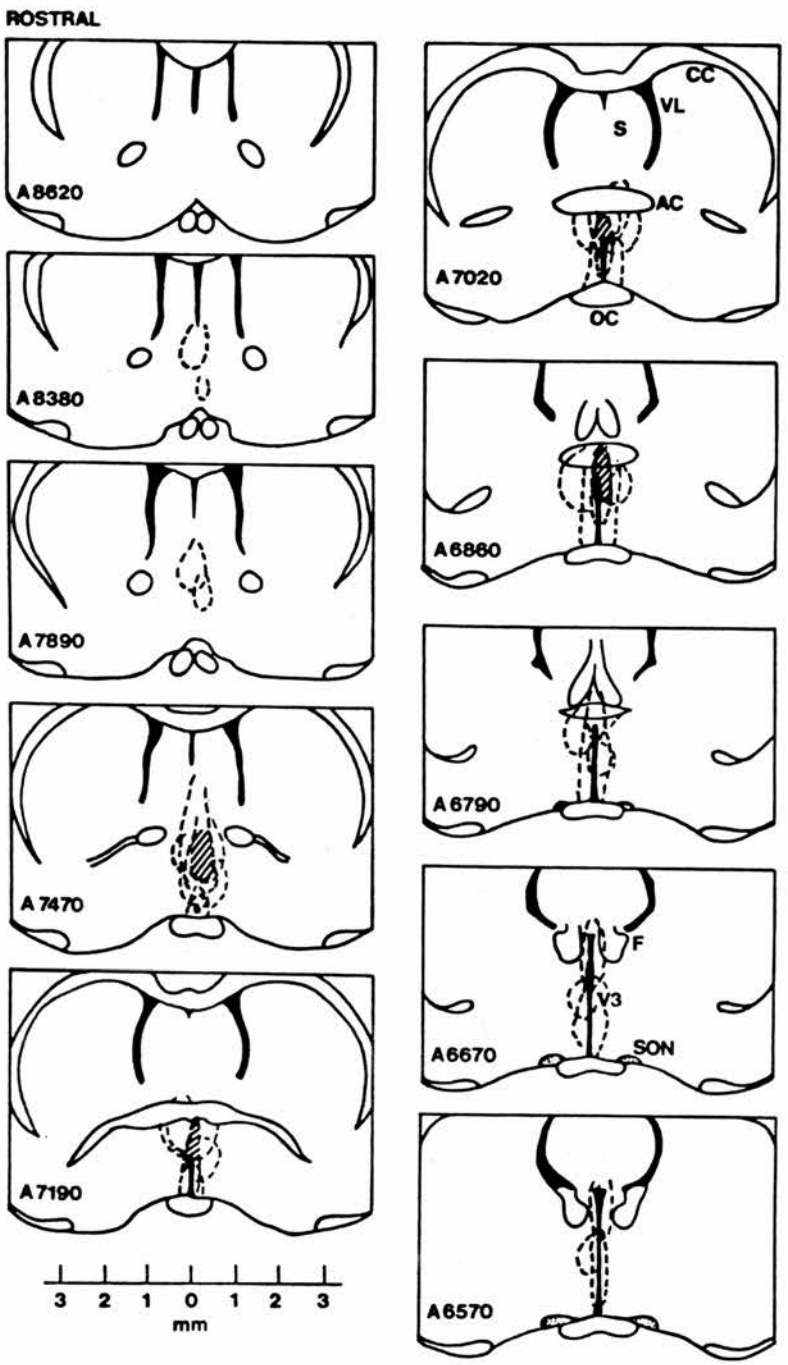

Figure 6.4.2.a Histological description of the anterior-posterior extent of AV3V lesions in the chronic i.c.v. vehicle-infused group (n=6). Lesions in individual rats are depicted with a dashed line on drawings of serial coronal sections of rat brain (after Konig and Klippel, 1963). The area common to all lesions in each plane is shaded.

The number at the bottom left of each section is the distance in μm anterior to the inter-aural plane.

Abbreviations: AC: anterior commisure; CC: corpus callosum; OC: optic chiasm; S: septum; F: fornix; SON: supraoptic nucleus; VL: lateral ventricle and V3: third ventricle.

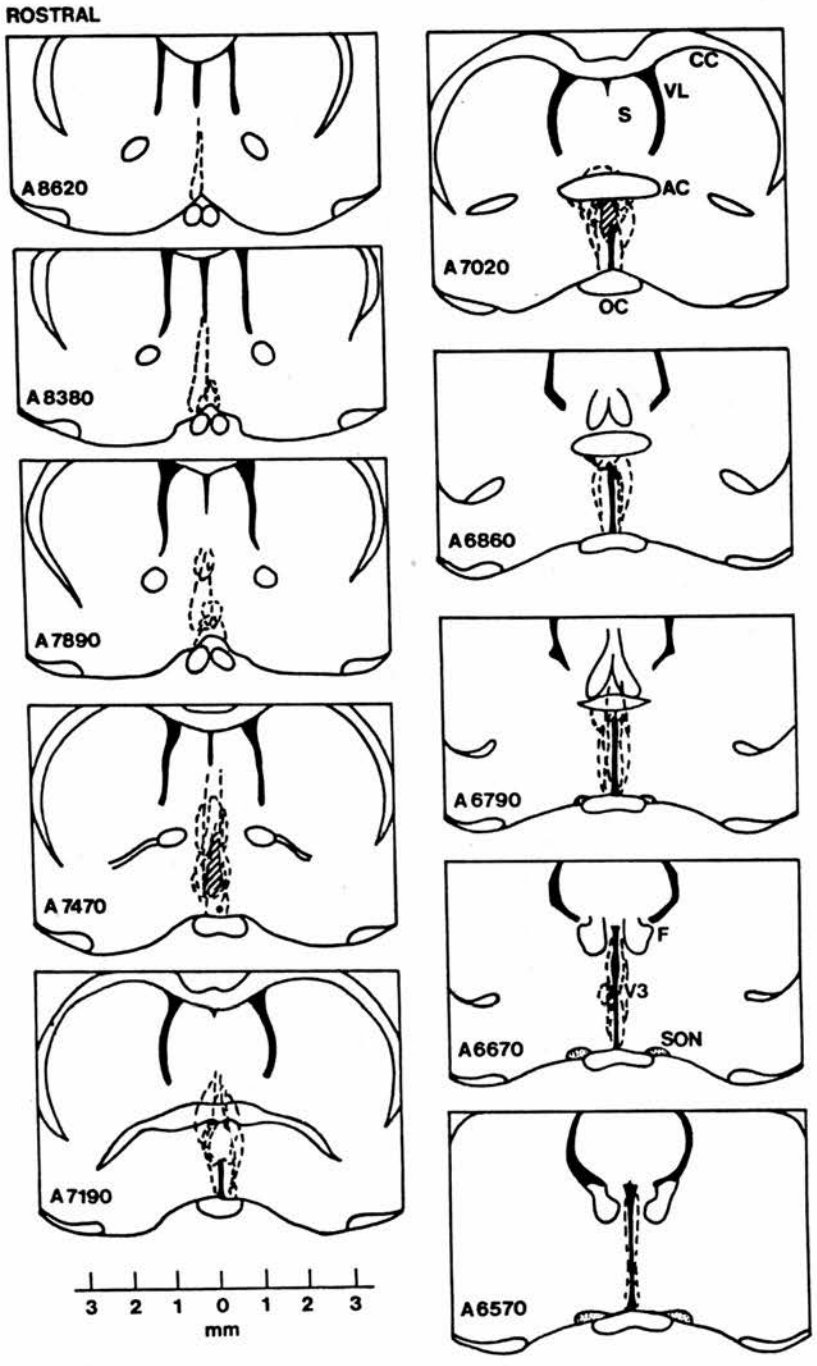

Figure 6.4.2.b Histological description of the anterior-posterior extent of AV3V lesions in the chronic i.c.v. morphine-infused group (n=6). Lesions in individual rats are depicted with a dashed line on drawings of serial coronal sections of rat brain (after Konig and Klippel, 1963). The area common to all lesions in each plane is shaded.

The number at the bottom left of each section is the distance in μm anterior to the inter-aural plane.

Abbreviations: AC: anterior commisure; CC: corpus callosum; OC: optic chiasm; S: septum; F: fornix; SON: supraoptic nucleus; VL: lateral ventricle and V3: third ventricle.

6.4.3. How does naloxone differentiate between the four treatment groups?

Data from the V/S group, can be compared with data previously reported (Bicknell et al, 1988a) for a control group of rats which were i.c.v. vehicle-infused and blood sampled immediately before and 6 min after i.v. naloxone (5mg/kg). The rise in plasma OT measured then was from 42.1 to 125.1 pg/ml, which is similar to that measured here, 67.3 to 251.1 pg/ml. The slight differences in protocol (i.e. sham-lesion procedure, number of blood samples) might account for the slightly higher value for post-naloxone OT concentration here although it is not clear whether the two values are significantly different anyway. This result points to a tonic opioid restraint on OT release which is revealed by naloxone. However, because OT neurones recorded from these control rats are not excited by i.v. naloxone (Bicknell et al, 1988a; Shibuki et al, 1988), the authors suggest that this rise in plasma OT is explained by antagonism of the actions of endogenous opioids upon OT release from nerve terminals located in the posterior pituitary. As discussed in the Introduction, Section I.2., naloxone antagonises endogenous opioids which appear to tonically inhibit oxytocin secretion from the neurohypophysis (Bicknell and Leng, 1982; Bicknell et al, 1988a). There are opioid receptors in the posterior pituitary which are probably exclusively the κ-type (Bunn et al, 1985; Herkenham et al, 1986; Stojilkovic et al, 1987; Sumner et al, 1990). Opioid receptors have been localised to pituicytes (Lightman et al, 1983b) and κ-opioid receptors found on neurosecretosomes (derived from nerve terminals in the posterior pituitary) are activated by the κ-receptor-selective agonist, U50,488H (Zhao et al, 1988b). Dynorphin, which is co-localised with vasopressin in neurosecretory neurones (Watson et al, 1982; Martin et al, 1983) is probably the endogenous ligand for the κ-receptor (Corbett et al, 1982). So, a part of the total amount of OT secreted after naloxone in each group is probably derived by antagonism of tonic endogenous opioid-mediated inhibition of OT secretion. The neurohypophysis was once proposed as the main site of action of morphine to inhibit OT secretion (Clarke et al, 1979), but more recent studies show this not to be so

(Coombes and Russell, 1989) so that even in morphine-dependent rats, naloxone's actions at the level of the posterior pituitary are likely to be only antagonism of endogenous opioids to which cross-tolerance does not develop (Bicknell et al, 1985b).

Comparison of the amount of OT liberated after naloxone in the V/S and the V/L groups highlights a difference brought about by acute AV3V lesion. OT concentration although elevated after naloxone in the V/L group, is much less so than in the V/S group (see Table 6.3.1.) in absolute terms (although the proportion by which OT secretion is raised after naloxone is not significantly different between the V/L and V/S groups (Wilcoxon test for independent samples)).

The AV3V region maintains a tonic excitatory input to the SON OT neurones under basal conditions (Russell et al, 1988). Stimulation of the AV3V region excites OT neurones in the SON (Russell et al, 1988). If the AV3V input is removed by ablation of the AV3V region, then the overall activity of the nucleus declines (Blackburn and Leng, 1987; Russell et al, 1988; Leng et al, 1989a) which can be expected to lead to a fall in the amount of OT secreted basally (see earlier Discussion) and conceivably after non-osmotic stimulation too because of the reduced excitability of the neurones. Such a fall in OT release has already been reported after chronic AV3V lesion (Russell et al, 1984; Gardiner et al, 1985) and acutely there is loss of responsiveness to osmotic stimulation (Blackburn et al, 1987) but not to suckling or parturition (Russell et al, 1988).

Perhaps therefore the shortfall in OT output in the V/L group after naloxone is explained simply by a decrease in electrical activity at the cell bodies of OT neurones in the SON and PVN which project to the posterior pituitary where consequently they secrete less OT under basal conditions with or without naloxone antagonism of endogenous opioid peptides. This idea is discussed further after consideration of the data from the chronic morphine-treated groups (see later Discussion).

However, it does not follow that all OT neuronal activity is abolished after AV3V lesion. The dramatic reduction in OT cell electrical activity in the SON after acute

ablation of the AV3V structures was not matched by an equal fall in OT measured in the plasma (Leng et al, 1989a). The authors explain this unexpected finding by suggesting that the magnocellular OT-secreting population of neurones in the PVN are not so critically involved in the component of osmotic responsiveness which is AV3V-mediated. This interpretation of results is supported by an early study which reported a weaker response from PVN OT neurones in terms of OT release after a systemic osmotic challenge compared to SON OT neurones (Brimble et al, 1978). The possibility arises therefore that whilst SON OT neurones are substantially affected by removal of the AV3V tonic drive, PVN OT neurones are not and help to maintain basal release of OT at the concentration measured in this study which was higher than would be expected in the virtual absence of electrical activity in the OT neurosecretory system suggested from studies in SON after AV3V lesion.

Some residual stimulation of OT neurones might exist after AV3V lesion as not all fibres arising from the SFO course through the AV3V region but travel laterally to terminate in the SON (Miselis, 1981; Lind et al, 1985; Swanson and Lind, 1986). The SFO is implicated in body fluid homeostasis (Gross, 1985; Thrasher, 1985) and may be an osmosensitive site (Buranarugsa and Hubbard, 1988; Sibbald et al, 1988). The SFO also contains opioid receptors (Mansour et al,1987; Sharif and Hughes, 1989; Conrath and Cupo, 1989) and opioid-sensitive neurones (Buranarugsa and Hubbard, 1979) and could therefore conceivably contribute to an integrated response to osmotic challenge, together with the OT and AVP neurones of the magnocellular nuclei.

Endogenous opioids and their receptors are present in or close to the magnocellular OT hypothalamic nuclei (Mansour, et al, 1986; Mansour et al, 1987; Tempel and Zukin, 1987; Sumner et al, 1990; see also General Introduction, Section I.2.). Application of opioids to OT neurones in vitro and in vivo causes a reduction in their firing rate (Muhlethaler et al, 1980; Pittman et al, 1980; Wakerley et al, 1983; Pumford et al, 1987; Bicknell et al, 1988a; Russell et al, 1989b; Leng et al, 1990;

see Chapters 1 and 2) and it is conceivable that endogenous opioids inhibit OT perikarya directly. Removal of the excitatory projection from the AV3V region could hypothetically allow any endogenous opioid inhibition to predominate. Given the many reports supporting a possible role for endogenous opioids in the regulation of OT secretion, this would be one scenario to explain the reduction in electrical activity of OT neurones after an AV3V lesion and the observed fall in plasma OT. A problem with the situation outlined above is that it infers that naloxone would not only antagonise opioid inhibition at the neurohypophysis but also at the cell bodies in the AV3V-lesioned rats, together resulting in a sizeable excitation of the OT system with an appropriately large increase in OT release but this appears not to be so. Endogenous opioids play an insignificant role, if any, in tonically inhibiting OT secretion by regulating central neuronal activity in urethane-anaesthetised virgin rats as it has been shown that naloxone cannot excite OT neurones in morphine-naive rats silenced by AV3V ablation (Leng and Russell, personal communication), nor does it affect OT neurones in intact rats stimulated by hyperosmolarity (Shibuki et al, 1988).

The most striking observation in the present study was the dramatic rise in plasma OT after naloxone in the M/S group. The rise from 65.3 to 1839.0 pg/ml OT compares closely with that reported in an earlier study in which a similar protocol, excepting the sham lesioning procedure, was used (44.7 to 1072.1 pg/ml, Bicknell et al, 1988a). This increase in OT output is far greater than that seen in the V/S group after naloxone (proportional increase in OT concentration expressed as time point 5 / time point 4: 33.7 vs. 5.9, M/S vs. V/S respectively, $P=0.02$, Wilcoxon test for independent samples) and the concentration of OT measured after naloxone is also significantly different between the V/S and M/S groups ($P<0.05$, Wilcoxon test for independent samples). This suggests that whilst tolerance has developed to chronic i.c.v. morphine as inferred by similar plasma OT concentration between groups in the first blood sample, so too has dependence upon i.c.v. morphine, as revealed after naloxone which precipitated withdrawal in terms of OT release (Bicknell et al, 1988a).

The increase in OT secretion is likely to be the result of the combined effects of increased neuronal firing rate (see Chapter 1) and antagonism of endogenous opioid peptide action at the posterior pituitary, as discussed above and as shown in the lactating morphine-dependent rat (Bicknell et al, 1988a).

The M/L group also responded to naloxone with a large rise in OT output from 24.3 to 326.3 pg/ml OT ($P<0.005$ vs pre-naloxone value, Student's paired one-tailed t-test). The plasma OT concentration after naloxone was greater than that seen in the V/L group ($P=0.005$, Wilcoxon test for independent samples) and the proportional increase in plasma OT was significantly greater in the M/L group compared with the V/L group (15.9 vs. 4.7 respectively, $P<0.01$, Wilcoxon test for independent samples). So, chronic i.c.v. morphine infusion has resulted in a greater responsiveness of the OT system to naloxone-evoked OT secretion, even after acute lesion of the AV3V region.

However, the sensitivity of the OT system to naloxone in the M/L group is much attenuated compared with that of the M/S group. This is unlikely to be the result of increased potency of naloxone at the level of the posterior pituitary as naloxone does not stimulate OT release more potently from isolated pituitaries derived from morphine-dependent rats, compared to control rats (Bicknell et al, 1985b). It seems therefore to be explained by antagonism of morphine at the level of the perikarya as is the case in the M/S group. Indeed, some restoration of electrical activity has been achieved with naloxone in acutely AV3V-lesioned morphine-dependent rats (Bicknell et al, 1987b) adding weight to this proposal.

Lesion of the AV3V region therefore diminishes the effectiveness of naloxone, but does not abolish its potency. So, although the M/L group show a greater responsiveness to naloxone compared with the V/L group, as discussed above, full potential sensitivity, as shown in the M/S group has been impaired by AV3V lesion. This might be because of the relatively low initial firing rate before naloxone in the M/S group as predicted by electrophysiological studies (Leng et al 1989a; Russell et al, in press) which even when stimulated would remain lower than the basal firing rate

of sham-lesioned controls. Given that the relation between firing rate and secretion of OT is not linear but that as firing rate increases, release is disproportionately increased (Bicknell, 1988), it is clear that the relative shortfall in firing rate of OT neurones in the lesioned group will lead to a much exaggerated impairment of OT release. It seems reasonable to assume that naloxone acted in the same direction on the electrical activity of OT neurones in both lesioned and unlesioned groups but given the discussion above, provoked quite different plasma concentrations of OT.

Opioid receptors in the SON have been reported both by ourselves (Sumner et al, 1990; see Chapter 5) and by others (Atweh and Kuhar, 1983; Clark et al, 1986; Mansour et al, 1986; Mansour et al, 1987; Tempel and Zukin, 1987) and therefore it follows that the component of opiate withdrawal which is not affected by AV3V lesion could be within the SON itself.

Another interpretation which does not exclude the former is that part of the withdrawal hypersecretion of OT is mediated by opioid receptors in the AV3V region or in areas which project to/through the AV3V region which become dependent upon morphine. Naloxone methyl bromide - a salt which does not cross the blood-brain barrier given i.v., elicits withdrawal in terms of OT secretion, albeit incomplete, suggesting that a site outside of the blood-brain barrier mediates in part, the expression of withrawal after naloxone (Leng et al, 1989b; the SFO contains opioid receptors, see above). We have reported μ-type opioid receptors in the MPN whose density decreases after chronic morphine treatment (Sumner et al, 1990; see Chapter 5). There are opioid receptors in other components of the AV3V region (Mansour et al,1987; Conrath and Cupo, 1989; Sharif and Hughes, 1989).

In conclusion therefore, naloxone has revealed that the expression of dependence upon morphine, by its precipitate withdrawal, is mediated partially at the supraoptic neurones and partially elsewhere in the hypothalamus - possibly in the AV3V region or in areas which project to or which project via the AV3V region to other critical sites involved in the centrally-mediated osmotic response . A conclusion cannot be drawn

from these data about what involvement the AV3V region might have in the development of tolerance to and dependence upon chronic i.c.v. morphine. This could be investigated by using rats AV3V-lesioned and implanted with an i.c.v. morphine infusion assembly simultaneously, 5 days before blood sampling with a protocol similar to that used here. Hypothetically this might reveal the part played by the AV3V region in the process of adaptation to chronic morphine, although in practice compensatory mechanisms would be expected to come into operation during the 5 day i.c.v. infusion period, possibly confusing the interpretation of these experiments.

Perhaps then, given these results, the appealing model of morphine tolerance and dependence occurring in a single cell type in isolation and explained in terms of changes in adenylate cyclase (Sharma et al, 1975a) does not transfer easily into the fully integrated neural networks of the CNS. In particular it does not model the OT neurosecretory system fully, which requires the functional integrity of other hypothalamic structures to fully express morphine dependence evidenced by the withdrawal syndrome after naloxone. Full withdrawal excitation may result from amplification of withdrawal within individual neurones by their synaptic connections with the final neurone (e.g. the oxytocin neurone), in the manner of a cascade.

It is difficult to tie the role of the AV3V region and the SON OT neurones to a possible physiological scenario in which tolerance to and dependence upon endogenous opioids might develop and at some point be withdrawn. I have speculated elsewhere about a possible build up of endogenous opioid tone before parturition which might be antagonised at a critical point (i.e. immediately prior to the expulsion of the first foetus) by an endogenous antagonist resulting in a surge in OT. I have not found satisfactory evidence in the literature to test such a hypothesis and so the elucidation of a physiological correlate of morphine dependence in the AV3V/SON axis remains a strong candidate for future research.

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Opiate Dependence in a Neuroendocrine Neurone System

J. A. Russell*, K. Pumford* and G. Leng†

*Department of Physiology, University Medical School, Teviot Place, Edinburgh EH8 9AG;
†A.F.R.C. Institute of Animal Physiology and Genetics Research, Babraham, Cambridge CB2 4AT, UK

ABSTRACT

Oxytocin secretion in the rat is inhibited by kappa-opioid receptor agonists acting in the posterior pituitary gland and by mu- and kappa-agonists (morphine and U50,488H) inhibiting the firing rate of oxytocin neurones in the supraoptic nucleus. U50,488H inhibits firing in the absence of Ca_o^{2+} in vitro. After intracerebroventricular infusion of morphine for 5 days larger doses of intravenous morphine are required to inhibit oxytocin neurones, indicating tolerance; naloxone-induced withdrawal stimulates oxytocin hypersecretion as a result of excitation of the firing of oxytocin neurones via mu receptors, partly outside the blood-brain-barrier, but partly independent of major excitatory inputs. The posterior pituitary shows neither tolerance nor dependence.

KEYWORDS

Dependence, morphine, oxytocin, tolerance, U50,488H.

OPIOIDS AND OXYTOCIN NEURONES

Opiates inhibit oxytocin secretion. For example, given to rats that have begun to give birth morphine dramatically slows parturition by decreasing oxytocin secretion in a naloxone-reversible fashion (Leng & Russell, 1989). Oxytocin secretion can be increased by opioid antagonists indicating that endogenous opioids restrain oxytocin secretion (Bicknell et al., 1988). Oxytocin is secreted by the posterior pituitary gland from the axon terminals of magnocellular neurones whose cell-bodies are in the supraoptic (SON) and paraventricular nuclei in the hypothalamus. All three classes of opioid peptide and μ- and k-subtype opioid receptors are associated with magnocellular oxytocinergic neurones (see Lincoln & Russell, 1986). Dynorphin produced by vasopressinergic neurones could act on adjacent perikarya or axon terminals of oxytocinergic neurones which may themselves produce met-enkephalin, and enkephalin-containing neurones and β-endorphin-containing terminals are found close to the magnocellular nuclei.

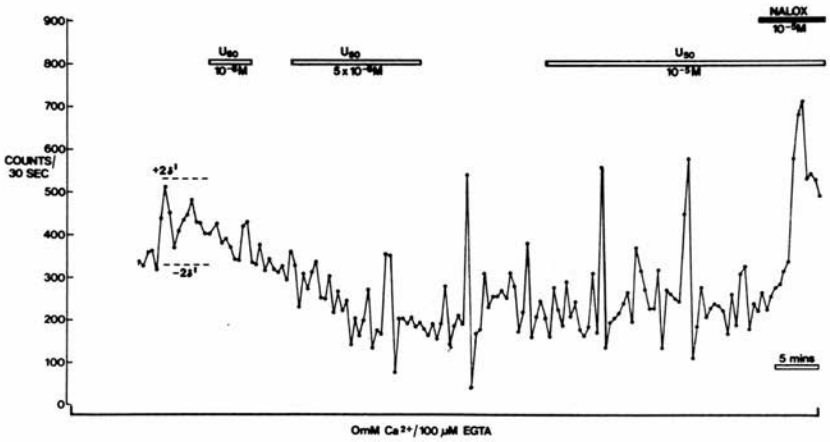

Fig. 1 Firing rate of a female rat SON neurone recorded extracellularly in a 450μm hypothalamic slice. Superfused with modified Yamamoto's medium containing zero Ca^{2+} and 100μM EGTA, which excites SON neurones. Despite the absence of Ca_o^{2+} U50,488H inhibited firing by ca. 60% (δ indicates control s.d.). Naloxone reversed the inhibition.

Opioid actions in this system can be investigated by measuring oxytocin secretion (an integral of the activity of all the oxytocin neurones) coupled with electrical stimulation of the posterior pituitary in vivo or in vitro; and by recording the electrical activity of single oxytocin neurones (characterized by their location and continuous, non-phasic firing pattern) since this is the major determinant of oxytocin secretion.

Sites of opioid action

Morphine does not act on the posterior pituitary in lactating rats; but the selective k-agonist U50,488H does inhibit oxytocin secretion evoked by electrical stimulation of the neurohypophysial stalk with an intravenous (i.v.) ID50 of ca.500 μg/kg (Coombes & Russell, 1988). Naloxone alone increases oxytocin secretion in vivo without affecting the firing rate of oxytocin neurones, and increases electrically-stimulated oxytocin secretion from the posterior pituitary in vitro (Bicknell et al., 1985, Bicknell et al., 1988). Thus in the posterior pituitary gland k- and not μ- receptors mediate inhibition of oxytocin secretion, and dynorphin is probably the endogenous ligand at this site.

Morphine, i.v. or i.c.v., inhibits in a naloxone-reversible fashion the spontaneous firing of SON oxytocin neurones and it inhibits the spontaneous or glutamate-stimulated activity of these neurones in hypothalamic slices (see Pumford et al., 1987, Leng & Russell, 1989). This explains how morphine inhibits oxytocin secretion. We have also found that the k-agonist, U50,488H, inhibits the firing of oxytocin neurones; the same neurone can be inhibited by both morphine and U50,488H. In the hypothalamic slice, U50,488H still inhibits, with unaltered potency, SON neurones excited by superfusion with medium containing zero Ca^{2+} and

100μM EGTA (Fig. 1), although k-agonists have been proposed to act on other neurones via inhibition of Ca^{2+} influx (see McFadzean, 1988).

Morphine tolerance and dependence

During 5 days of continuous i.c.v. infusion of morphine (up to 50μg/h from a subcutaneous osmotic minipump), normal function of oxytocin neurones returns indicating tolerance (Bicknell et al., 1988, Rayner et al., 1988). Electrically-stimulated oxytocin secretion from the posterior pituitaries of such rats is still enhanced by naloxone, indicating lack of cross-tolerance to the k-receptor mechanism in the posterior pituitary (Bicknell et al., 1985). The median threshold cumulative dose of i.v. morphine required to inhibit the spontaneous activity of SON oxytocin neurones was 25-fold greater in 13 chronic morphine-treated rats compared with 7 controls ($p = 0.0035$; median dose 60μg/kg in controls). The maximum effect (100% inhibition) was unaltered. These data are consistent with a decrease in the number of spare opioid receptors on oxytocin neurones or their inputs.

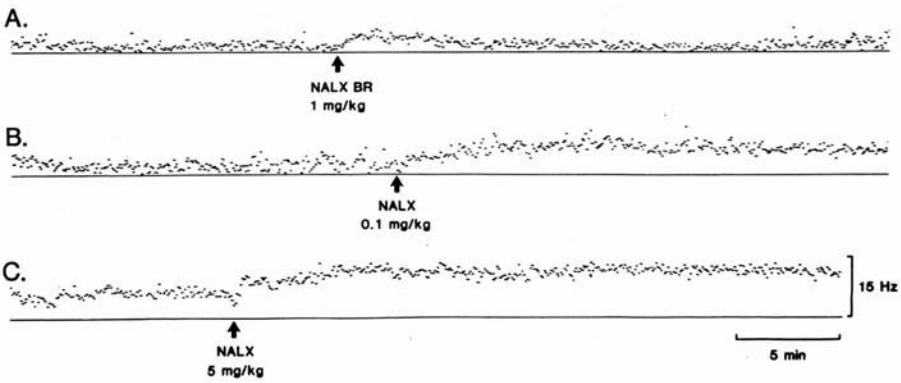

Fig. 2 Firing rate of a SON putative oxytocin neurone in a morphine-dependent rat anaesthetized with urethane (1.25g/kg i.p.). Naloxone methylbromide i.v. (NALX BR) excited the cell, indicating withdrawal at opioid receptors outside the blood-brain-barrier. Naloxone HCl (NALX) was more potent and increasing doses produced graded effects.

Dependence, revealed by excitation of oxytocin secretion by naloxone to a level not seen before opioid treatment, is evident in morphine-treated rats (Bicknell et al., 1988). Naloxone has no greater effect on oxytocin secretion from the isolated posterior pituitaries of morphine-dependent rats compared with controls (Bicknell et al., 1985). However, the firing-rate of SON oxytocin neurones in morphine-dependent, but not control, urethane-anaesthetized rats is increased by 360% after naloxone(5mg/kg i.v.) (Fig. 2) and this excitation explains oxytocin hyper-secretion during withdrawal (Bicknell et al., 1988). The ED50 for naloxone's effect on firing rate is ca. 50μg/kg. Together with a lower potency of MR2266 (Bicknell et al., 1987) these findings indicate that μ-receptors mediate the withdrawal excitation. Excitation of oxytocin neurones in morphine-dependent rats by i.v. naloxone methylbromide indicates some involvement of μ-receptors outside the blood-brain barrier, perhaps in a

circumventricular organ (Fig. 2). However, an acute electrolytic lesion of the AV3V region, which relays information from the subfornical organ and organum vasculosum of the lamina terminalis (Russell et al., 1988) merely attenuates withdrawal excitation of oxytocin secretion (unpubl.). These and previous studies (Bicknell et al., 1988) on the role of synaptic input indicate that a major site of morphine withdrawal is on or close to the oxytocin neurones themselves.

Morphine tolerance and dependence in the magnocellular oxytocin system demonstrate potential involvement of endogenous opioids not only in acutely inhibiting secretion but also in resetting the sensitivity to other inputs.

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Actions of the κ-type opioid receptor agonist U50,488H on oxytocin neurones in the supraoptic nucleus (SON) in normal and morphine-tolerant urethane-anaesthetized rats

By G. Leng*, K. Pumford and J. A. Russell. *Department of Physiology, University Medical School, Teviot Place, Edinburgh, EH8 9AG and *Department of Neuroendocrinology, AFRC Institute of Animal Physiology and Genetics Research, Cambridge, CB2 4AT*

The terminals of magnocellular oxytocin neurones in the posterior pituitary gland are inhibited by opioids acting through κ-type receptors (Coombes & Russell, 1988). The firing of action potentials by the cell-bodies of these neurones which is the major determinant of oxytocin secretion, is inhibited by the μ-type opioid receptor agonist, morphine (Leng & Russell, 1989). But the SON contains κ-type receptors (Mansour *et al.* 1988), so we have investigated whether the firing-rate of oxytocin neurones is affected by the κ-receptor selective agonist U50,488H.

Adult virgin female Sprague–Dawley rats were anaesthetized with urethane (1·25 g kg^{-1}, I.P.) and via a ventral surgical approach a SON was exposed for extracellular recording (0·15 M-NaCl-filled glass micro-electrodes, 15 to 50 MΩ) of the spontaneous activity of continuous, putative oxytocinergic neurones, identified antidromically by stimulation of the exposed neurohypophysial stalk (Bicknell *et al.* 1988). Firing-rate averaged in 30 s bins was analysed over 10 min epochs. Drugs were injected via a jugular vein. Of eight neurones tested, seven were inhibited by doses of U50,488H above 1 μg kg^{-1}; the range of threshold cumulative dose to inhibit firing was 1 to 6661 μg kg^{-1} ($n = 7$ cells). All neurones tested with morphine sulphate, up to 6661 μg kg^{-1}, were inhibited; the median threshold cumulative dose was 20 μg kg^{-1}, ($n = 8$ cells). In rats given intracerebroventricular morphine infusion for 5 days (Bicknell *et al.* 1988), the median threshold inhibitory dose for I.V. morphine was increased to 1660 μg kg^{-1} ($n = 11$ cells, $P = 0{\cdot}02$ *vs.* controls, Kolmogorov–Smirnov), but the threshold inhibitory dose for I.V. U50,488H was unaltered. In both control and morphine-tolerant rats, low doses of U50,488H (1 μg kg^{-1}) excited 6/18 supraoptic oxytocin neurones: in these cells mean ± S.E.M. firing-rate increased from 3·79 ± 0·85 Hz to 5·16 ± 0·82 Hz ($P < 0{\cdot}005$, paired t test 6 cells). Thus both κ- and μ-type opioid receptor agonists can inhibit the firing of oxytocin neurones; after chronic morphine exposure sensitivity to morphine but not U50,488H is decreased; at low doses U50,488H is excitatory indicating inhibition of an inhibitory input to oxytocin neurones.

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OPIOID RECEPTOR SUBTYPES IN THE SUPRAOPTIC NUCLEUS AND POSTERIOR PITUITARY GLAND OF MORPHINE-TOLERANT RATS

B. E. H. SUMNER,* J. E. COOMBES, K. M. PUMFORD and J. A. RUSSELL
Department of Physiology, University Medical School, Teviot Place, Edinburgh EH8 9AG, U.K.

Abstract—Morphine, given acutely, inhibits oxytocin secretion in adult female rats, but chronic intracerebroventricular infusion for five to six days induces tolerance and dependence in the mechanisms regulating oxytocin secretion. One explanation for tolerance could be that there is a loss of opioid receptors.

To test this hypothesis cryostat sections of selected brain regions and the pituitary, from six control and six intracerebroventricular morphine-infused rats, were processed for quantitative *in vitro* receptor autoradiography. [^{3}H]Etorphine or [^{3}H](−)-bremazocine were used as ligands, and DAGO, DPDPE and U50,488H as selective displacers from μ-, δ-, and κ-receptors, respectively. Control incubations had naloxone determined specificity. The supraoptic nucleus (site of oxytocin-secreting magnocellular perikarya) contained both μ- and κ-receptors in control rats (mean ± S.E.M. binding of μ-selective [^{3}H]etorphine was 91.8 ± 25.4 fmol/mg of tissue, and of κ-selective [^{3}H](−)-bremazocine was 130.4 ± 25.6 fmol/mg). Chronic morphine treatment caused a 83.9% decrease in binding in μ-selective conditions ($P < 0.05$), but no significant change in κ-selective binding. In the median preoptic nucleus (which projects to the supraoptic nucleus) mean ± S.E.M. binding of [^{3}H]etorphine decreased by 77.0% ($P < 0.01$) in chronic morphine-treated rats, from the control value of 76.2 ± 9.8 fmol/mg of tissue. In the posterior pituitary gland (site of the terminals of the oxytocin-secreting magnocellular perikarya) binding with [^{3}H](−)-bremazocine in controls was over 90% lower than in the supraoptic nucleus. No changes followed chronic morphine treatment.

Thus chronic morphine exposure reduces the numbers of available μ-receptors in the supraoptic nucleus, and of opioid receptors in the median preoptic nucleus, perhaps accounting for morphine-tolerance in relation to oxytocin secretion.

The inhibitory effects of opiates on oxytocin secretion were first demonstrated only a decade ago.[18] Since then inhibitory actions of both exogenous opiates and endogenous opioids have been shown in a range of studies on the oxytocin system, mainly in the rat (e.g. see Refs 20, 48, 50). Morphine acts centrally by inhibiting the firing rate of continuously firing (putatively oxytocin-secreting) neurons in the supraoptic nucleus (SON).[6,58] Endogenous opioids act also on oxytocin-releasing nerve terminals in the posterior pituitary (PP) gland.[3,5,6] Opioids can also inhibit vasopressin secretion *in vivo*,[2] but their effects on perikaryal electrical activity are inconsistent,[58] and their action on vasopressin release from the PP is less striking,[3–5,17,58] except for a dose-dependent inhibition by ethylketocyclazocine,[55] and U50,488H.[60,61]

The actions of opioids in brain are mediated by three principal opioid receptor subtypes, classified by agonist selectivity.[1,22,63] Mansour *et al.*[34–36] and Tempel and Zukin[57] have surveyed the distribution of μ-, δ- and κ-type opioid receptors in the rat brain, using *in vitro* receptor autoradiography. In the SON, κ-type receptors but not μ- or δ-receptors were found, yet both μ- and κ-opioid receptor agonists inhibit the electrical activity of SON neurons.[49] In the PP, κ-type receptors predominate, with few μ-receptors and no δ-receptors;[12,25] κ-selective, but not μ- or δ-selective, agonists inhibit oxytocin secretion at this site.[7,19]

Chronic morphine exposure leads to tolerance (loss of opiate effect, or a requirement for a larger dose to achieve the initial effect) and dependence (revealed as hyperexcitation on withdrawal). Both changes are seen in the mechanisms regulating oxytocin secretion in the rat after i.c.v. infusion of morphine for five days.[6,43,54] In contrast, dependence does not develop in the vasopressin system.[6]

One possible explanation for opioid tolerance is that the number of opioid receptors on tolerant neural elements diminishes. Previous *in vitro* autoradiographic studies on cryostat sections of morphine-tolerant rat brain found either no change in total opioid receptor numbers,[21] or an up-regulation of μ-receptors,[10] but with no observations on the SON

*To whom correspondence should be addressed.

Abbreviations: AV3V region, region anterior and ventral to the third ventricle; DAGO, Tyr-D-Ala-Gly-MePhe-NH(Ch$_2$)$_2$OH, a μ-type opioid receptor agonist; DPDPE, [D-Pen^2, D-Pen^5]-enkephalin, a δ-type opioid receptor agonist; G_i, inhibitory guanine nucleotide-binding protein; MPN, median preoptic nucleus; PP, posterior pituitary; SON, supraoptic nucleus; U50, 488H *trans*-(±)-3,4-dichloro-*N*-methyl-*N*-(2-[1-pyrrolidinyl]-cyclohexyl) benzeneacetamide, methane sulphonate salt, a κ-type opioid receptor agonist.

or PP. Studies on ^{3}H-ligand-binding to other brain preparations have also yielded conflicting results in morphine-tolerance.[8,27,28,42,45,46,52,56,59] However, most of these studies did not distinguish between opioid receptor subtypes, and none focused on the SON and PP. The aims of the present study were to measure the densities of μ- and κ-opioid receptor subtypes in the SON and PP in normal and morphine-tolerant female rats, to try to explain morphine-tolerance in relation to oxytocin neurons.

We also measured opioid receptor density in the median preoptic nucleus (MPN), which is part of the region anterior and ventral to the third ventricle (AV3V region) that projects to the supraoptic nuclei,[47] and might contribute to tolerance of the oxytocin system.

EXPERIMENTAL PROCEDURES

Materials

The following were used as probes for opioid receptors *in vitro*. (i) [^{3}H]Etorphine (50 Ci/mmol ≡ 1.85 TBq/mmol Amersham);[12,33] (ii) [^{3}H](−)-bremazocine (21.3 Ci/mmol ≡ 0.79 TBq/mmol, NEN).[9,38]

The following were used as competitive displacers of the ^{3}H-ligands from individual opioid receptor subtypes. (i) Tyr-D-Ala-Gly-MePhe-NH(CH_2)$_2$ OH, or DAGO (cat. No. PB2000A, Cambridge Research Biochemicals), for displacement from μ-receptors;[34] (ii) [D-Pen^2, D-Pen^5]-enkephalin, or DPDPE (cat. No. E-0260, Sigma), for displacement from δ-receptors;[34,40] (iii) *trans*-(±)-3,4-dichloro-*N*-methyl-*N*-(2-[1-pyrrolidinyl]cyclohexyl) benzeneacetamide, methane sulphonate salt, or U50,488H, (cat No. D-0908, Sigma) for displacement from κ-receptors.[16,38] To test specificity, naloxone hydrochloride (cat. No. N-7758, Sigma) was used.

Intracerebroventricular infusion

Adult virgin female albino Sprague–Dawley rats were implanted stereotaxically under ether anaesthesia with a cannula in a lateral cerebral ventricle, for continuous infusion from a subcutaneous osmotic minipump (Alzet, 2001) of either morphine sulphate (10 μg/h for 40 h increasing to 20 μg/h, and then 50 μg/h, each for 40 h), or vehicle (sterile, pyrogen-free distilled water, 1 μl/h).[43] There were six rats in each group, and the mean ± S.E.M. body weights were 274.8 ± 5.1 g in the morphine group and 288.8 ± 8.7 g in the vehicle group.

Tissue preparation

After five days of i.c.v. infusion, the rats were decapitated, their brains removed, and from each a thick coronal slice was cut extending from approximately 1 mm anterior to the optic chiasma, to the posterior limit of the mammillary body. Each slice was frozen onto a pre-cooled layer of Tissue-Tek II OCT compound (Miles Laboratories) on a cryostat chuck, in crushed dry ice. The pituitary gland containing the PP was frozen similarly after orientation for coronal sectioning.

Cryostat sections were cut at 10 μm, and thaw-mounted onto acid-cleaned, chrome alum–gelatine-subbed slides. From each rat for each incubation category there were four sections containing two SON profiles each, four sections of the MPN, and four sections of the PP. Mounted sections were stored at −20°C for up to three weeks before incubation.

Detection of opioid receptors

An incubating medium containing a broad-spectrum ^{3}H-ligand (2 nM), without additional agonists, was used for total opioid receptors, but for receptor subtypes two unlabelled selective agonists were included (2 μM each)[57] with the ^{3}H-ligand, with the intention of competitively displacing the ^{3}H-ligand from all but the third receptor subtype. In pilot studies, higher concentrations of displacer (up to 50 μM) did not reduce ^{3}H-ligand-binding further. The autoradiographic images from different incubation categories (see Results) suggested that at 2 μM the displacers were effective and selective in the protocol for either ^{3}H-ligand.

[^{3}H]Etorphine (with displacers) was used for μ-receptors in brain, but [^{3}H](−)-bremazocine (with displacers) was used for κ-receptors, since pilot studies showed more binding by [^{3}H](−)-bremazocine than by [^{3}H]etorphine in brain sections when DAGO and DPDPE were present. Only [^{3}H](−)-bremazocine (with or without displacers) was used for the pituitary, since we obtained negligible specific labelling with [^{3}H]etorphine, even after long exposure. Neither ^{3}H-ligand gave measurable binding in the presence of DAGO and U50, 488H at either site, or elsewhere in brain sections, so quantitative analysis of this category was omitted. Control sections were incubated with 2 nM ^{3}H-ligand and 2 μM naloxone hydrochloride to antagonize all binding to opioid receptors. In pilot studies, higher concentrations of naloxone (up to 20 μM) did not further reduce binding. Further control sections were incubated with ^{3}H-ligand containing all three displacers to control for possible direct chemical interaction between naloxone and [^{3}H]etorphine, and this reduced binding like naloxone; the effects of naloxone were used to calculate specific binding (see below). Other control sections were incubated without ^{3}H-ligand to test for chemography. None of these sections produced autoradiographic images. The incubation categories and protocols are shown in Tables 1 and 2. Sections were brought to room temperature and then preincubated to detach and wash out endogenous opioid or exogenous opiate. We used procedures optimized for each ^{3}H-ligand by others;[9,12] Na^+ was not included in the incubating medium with [^{3}H]etorphine to seek to distinguish high/low affinity binding to μ-receptors.[13] The preincubating and incubating solutions, made up in deionized double-distilled water, were applied as a 40- or 20-μl puddle to each brain or pituitary section, respectively, on slides in moist chambers. After ice-cold immersion washes, the slides were air-dried, and stored desiccated at 0–4°C for less than 48 h before autoradiography. All the slides from a pair of control and morphine-treated rats were processed identically at the same time.

Autoradiography

Slides at room temperature were apposed to Hyperfilm-^{3}H (Amersham), keeping similarly incubated slides from each pair of rats adjacent. A 5-μm section of a tritium standard (^{3}H-microscales, Amersham) was applied to each film. The loaded films were clamped between mirror-coated glass plates with adhesive tape, sealed in light-tight wrappers, and exposed in the dark at 0–4°C.

The optimum exposure periods (determined empirically to produce grain densities over tissue that were below saturation for all positive incubations) were nine weeks for brain sections and 16 weeks for pituitary sections. The exposed films were separated from the slides, developed at 18°C in Kodak D19 developer (5 min), rinsed in tap water, fixed in Ilford Hypam rapid fixer (two changes, 5 min each, of 1:4 fixer:tap water), washed for 30 min in running water, then air-dried. The sections were fixed for 10 min in acetic acid:absolute ethanol:commercial formalin (1:17:2 by volume), dehydrated, rehydrated, stained for 15 min in 1% aqueous Cresyl Fast Violet, dehydrated, cleared, and mounted in DPX.

Image analysis

Pieces of film were first cut out and attached to slides.

Subjective assessment. Autoradiographs were matched with the stained sections in a Wild M3 binocular dissecting

Table 1. Incubation categories for the detection of opioid receptor subtypes in the different tissue regions

Incubation category	Intended selectivity for different opioid receptor subtypes	Tissue region(s) probed
[^{3}H]Etorphine alone (without displacers)	All subtypes (non-selective)	SON and MPN
[^{3}H]Etorphine + DPDPE + U50,488H	μ-receptor selective	SON
[^{3}H]Etorphine + naloxone	No naloxone-sensitive opioid receptors detected	SON and MPN
[^{3}H]Etorphine + DAGO + DPDPE + U50,488H	No μ-, δ-, or κ-receptors detected	SON and MPN
No ^{3}H-ligand, but [^{3}H]etorphine protocol	No receptors of any kind detected	SON and MPN
[^{3}H](−)-Bremazocine alone (without displacers)	All subtypes (non-selective)	SON and PP
[^{3}H](−)-Bremazocine + DPDPE + U50,488H	μ-receptor-selective	PP
[^{3}H]−)-Bremazocine + DAGO + DPDPE	κ-receptor-selective	SON and PP
[^{3}H](−)-Bremazocine + naloxone	No naloxone-sensitive opioid receptors detected	SON and PP
[^{3}H](−)-Bremazocine + DAGO + DPDPE + U50,488H	No μ, δ-, or κ-receptors detected	SON and PP
No ^{3}H-ligand, but [^{3}H](−)-bremazocine protocol	No receptors of any kind detected	PP

microscope (magnification: ×16), to identify appropriate brain regions.[41] Regional silver-grain density was graded on a subjective scale of 0, +, ++, +++, and ++++ (none, weak, moderate, moderately strong, strong).

Quantitative assessment of autoradiographs. The boundaries of the SON, MPN, and PP profiles were first scored with a needle-point on the film under the dissecting microscope. Silver-grain density in these regions was measured with a Joyce–Loebl μMagiscan image-analysing computer, receiving video input from a Philips black and white Video 40 camera (with a Newvicon tube) mounted on a Vickers M17 microscope.[54] Microscope magnification was ×10 (objective), and ×1.6 (intermediate lens); the video monitor scale factor was 1.1878 μm per pixel. The rectangular counting frame (201.63 × 133.04 μm) was set to be smaller than the average area of the SON, to make measurements in the dorsal SON, where oxytocin perikarya predominate.[44,54] The same counting frame was used for both MPN, and for the PP where separate central and peripheral measurements were made because more opioid receptors have been found in the oxytocin terminal-rich periphery.[9,25] Silver-grain density was calculated from the fraction:

$$\frac{\text{total area of silver deposit}}{\text{total area of counting frame}},$$ as justified previously.[54]

Grain densities over tissue were corrected by subtraction of a background count (measured over nearby tissue-free film). For each rat the mean grain density was determined for each tissue region (eight, four and four images of SON, MPN, or PP, respectively) in each incubation category. The mean grain density representing specific binding to opioid receptors was obtained by subtraction of the mean grain density over the corresponding naloxone control sections, if significantly above background (paired t-test). Then, since tissue from morphine- and vehicle-treated rats was co-processed in pairs, the mean grain densities in each incubation category were compared by the paired t-test.

For conversion to absolute values, grain densities were measured similarly, in triplicate, and backgrounds subtracted, for each step of the standard scale on each sheet of film. A mean standard curve of grain density against radioactivity in terms of its brain–gray matter–tissue equivalent (calibrated by Geary and Wooten for Amersham), was computed for the SON and MPN analysis (nine weeks exposure) and for the PP analysis (16 weeks exposure), and fitted to a power function ($y = 0.27\,x^{0.23}$ and $r = 0.97$, for the SON and MPN standard curve, and $y = 0.34\,x^{0.14}$ and $r = 0.96$, for the PP standard curve). Tissue radioactivity values (nCi/mg of tissue), were then calculated from the mean grain densities, and were converted to fmol of ^{3}H-ligand bound per mg of tissue, by reference to the specific activity of the ^{3}H-ligand. Specific binding to opioid receptors was obtained by subtracting binding in the presence of naloxone.

I.c.v. morphine and control group mean ^{3}H-ligand-binding values were compared by Student's t-test.

Preincubation tests

To test the efficiency of the preincubation wash procedure in detaching opioids or opiates from the tissue, brain sections were preincubated for 0, 1, 10, 15, 30, 45, 60, 75, or 90 min, incubated with [^{3}H]etorphine (μ-selective for SON; non-selective for MPN), and then processed and analysed as above.

Blood plasma analysis

Trunk blood was collected from each rat into heparinized tubes, plasma separated immediately by centrifugation, and stored at −20°C. Measurements were made of (i) oxytocin concentration by radioimmunoassay (Higuchi *et al.*[26]) to assess tolerance; (ii) glucose concentration (using a Boehringer Kit; Glucosa GOD-Perid) since morphine raises blood glucose concentration, but tolerance develops;[23] (iii) osmolality and sodium concentration[43] to assess hydromineral state. Student's t-test was used to compare treatment groups.

RESULTS

Supraoptic nucleus

Subjective assessment. Representative autoradiographs of the SON in a control rat are shown in Fig. 1. Using [^{3}H]etorphine alone the SON was moderately strongly labelled (+++), as was adja-

Table 2. Protocols used for the ^{3}H-ligands

^{3}H-ligand	Preincubation	Incubation	Washes	Reference
[^{3}H]Etorphine (± displacers)	15 min at room temperature in 0.05 M Tris–HCl buffer (pH 7.4) containing 0.1 M NaCl and 50 μM Gpp(NH)p	1 h at room temperature in 0.05 Tris–HCl buffer (pH 7.4) containing 0.19 M sucrose, and 2 nM [^{3}H]etorphine (± 2 μM displacers)	1 min in each of four successive baths of ice-cold 0.05 M Tris–HCl buffer (pH 7.4) containing 0.19 M sucrose; then a quick dip in ice-cold deionized double-distilled water to remove salts	After Bunn *et al.*[12] (but with modified washes and displacers)
[^{3}H](−)-Bremazocine (± displacers)	30 min at 0–4°C in 0.015 M potassium phosphate buffer (pH 7.4) containing 0.15 M NaCl and 0.1% BSA	3 h at 0–4°C in 0.05 M potassium phosphate buffer (pH 7.4) containing 0.4 M NaCl, 0.1% BSA, and 2 nM [^{3}H](−)-bremazocine (± 2 μM displacers)	1 min in each of four successive baths of ice-cold 0.05 M potassium phosphate buffer (pH 7.4) without additives, before the final quick rinse in ice-cold deionized double-distilled water	After Brady and Herkenham[9] (but with the addition of displacers)

BSA, bovine serum albumin; Gpp(NH)p, 5′-guanylylimidodiphosphate.

cent gray matter in the hypothalamus and thalamus. White matter gave only a feeble image (0 to +). The strongest labelling was in patches in the caudate–putamen, and in the amygdala (+ + + +). Elsewhere in the caudate–putamen, amygdala, and cerebral cortex, labelling was moderate (+ +). The pattern and intensity of labelling was similar in sections probed with [^{3}H]etorphine and DPDPE and U50,488H. Sections probed in the presence of DAGO and U50,488H gave only weak images (0 to +) in the SON and elsewhere.

Sections probed with [^{3}H](−)-bremazocine alone gave strong images in which the pattern of labelling resembled that shown by [^{3}H]etorphine, except that the cerebral cortex and caudate–putamen were more heavily labelled (+ + to + + +). The SON was moderately strongly labelled (+ + +). Sections probed with [^{3}H](−)-bremazocine and DAGO and DPDPE gave moderate images in the SON and elsewhere in the hypothalamic gray matter (+ +). The intense patches in the caudate–putamen and the amygdala, probed with either ^{3}H-ligand alone, or with [^{3}H]etorphine and DPDPE and U50,488H, were absent from sections probed with [^{3}H](−)-bremazocine and DAGO and DPDPE.

Specificity control sections, incubated with ^{3}H-ligand either with naloxone, or with all three unlabelled displacers, gave extremely faint images.

In morphine-treated rats, the pattern of labelling was similar to control rats, but there was a decrease in intensity in sections incubated with [^{3}H]etorphine and DPDPE and U50,488H.

Quantitative assessment. The results for the SON are shown in Table 3, and Fig. 2. Specific (naloxone-displaceable) binding was over 97% of the total binding in SON sections probed by ^{3}H-ligand alone.

The grain density over the SON incubated with [^{3}H]etorphine alone was reduced by 26.9% in the chronic morphine group ($P < 0.05$; Student's paired t-test). With [^{3}H]etorphine, DPDPE and U50,488H the decrease was 46.9% ($P < 0.02$). There was no significant difference between morphine- and vehicle-treated rats for any other incubation category. In the SON of control and morphine-treated rats the group mean (± S.E.M.) specific binding of [^{3}H]etorphine alone was 102.0 ± 10.5 and 51.7 ± 25.4 fmol per mg of tissue, respectively (not significant). Over SON profiles probed by [^{3}H]etorphine and DPDPE and U50,488H, mean binding was 91.8 ± 25.4 and 14.8 ± 7.5 fmol per mg of tissue in the control and morphine groups, respectively (83.9% decrease, $P < 0.05$, Student's t-test).

Specific binding of [^{3}H](−)-bremazocine alone in the SON was not significantly altered by i.c.v. morphine treatment. In the control and morphine groups the means were 125.7 ± 35.0 and 70.9 ± 24.8 fmol per mg of tissue, respectively. Similarly, treatment group means for SON probed with [^{3}H](−)-bremazocine and DAGO and DPDPE showed no significant difference between control and morphine groups

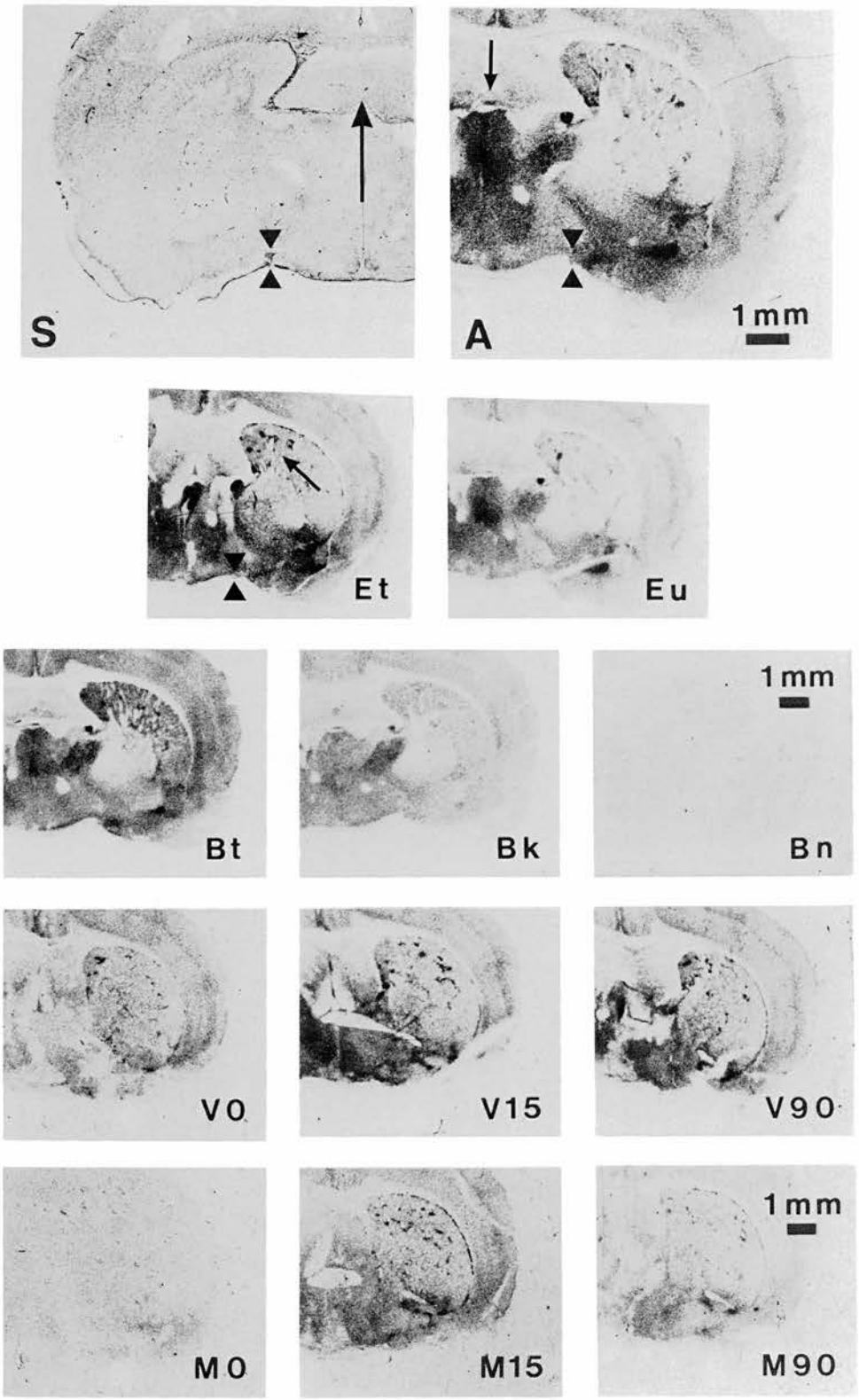

Fig. 1. (Top row) S is part of a 10-μm coronal section of rat brain, incubated with [^{3}H]etorphine, apposed to Hyperfilm-^{3}H for nine weeks, then fixed in acetic acid–ethanol–formalin, and stained with Cresyl Fast Violet. The SON is the small dark-staining area between the black triangles. The upward-pointing arrow is on the midline. A is the autoradiographic image corresponding to S, processed on the Hyperfilm-^{3}H after separation of S from the film. The position of the SON is indicated as in S, and the downward-pointing arrow is on the midline. Magnification × 6. (Second and third rows) Autoradiographic images of sections incubated with [^{3}H]etorphine alone (Et), or with [^{3}H]etorphine and DPDPE and U50,488H (Eu), or with [^{3}H](−)-bremazocine alone (Bt), or with [^{3}H](−)-bremazocine and DAGO and DPDPE (Bk), or with [^{3}H](−)-bremazocine and naloxone to antagonize all binding to opioid receptors (Bn). In [^{3}H]etorphine alone (Et), the SON is between the black triangles, and the arrow indicates intense patches of labelling in the caudate–putamen. For further neuroanatomical details, see Paxinos and Watson.[41] Magnification × 4. (Fourth and fifth rows) Autoradiographic images of sections from an i.c.v. vehicle-infused rat (V), and an i.c.v. morphine-infused rat (M), incubated with [^{3}H]etorphine and DPDPE and U50,488H following 0, 15, or 90 min preincubation as indicated. Magnification × 4.

Table 3. Treatment group means (±S.E.M.) of mean grain densities in autoradiographs of the supraoptic nucleus, median preoptic nucleus and posterior pituitary

Tissue region	Incubation category	Vehicle-treated animal group ($n = 6$)	Morphine-treated animal group ($n = 6$)	Morphine-treated versus vehicle-treated (Student's paired t-test)
SON	[^{3}H]Etorphine alone	0.38 ± 0.01	0.28 ± 0.04	$P < 0.05$
	[^{3}H]Etorphine + DPDPE + U50,488H	0.37 ± 0.02	0.20 ± 0.04	$P < 0.02$
	[^{3}H](−)-Bremazocine alone	0.32 ± 0.02	0.27 ± 0.03	n.s.
	[^{3}H](−)-Bremazocine + DAGO + DPDPE	0.32 ± 0.01	0.30 ± 0.02	n.s.
MPN	[^{3}H]Etorphine alone	0.36 ± 0.01	0.25 ± 0.02	$P < 0.05$
PP	[^{3}H](−)-Bremazocine alone	0.18 ± 0.04	0.19 ± 0.05	n.s.
	[^{3}H](−)-Bremazocine + DAGO + DPDPE	0.13 ± 0.05	0.15 ± 0.03	n.s.
	[^{3}H](−)-Bremazocine + DPDPE + U50,488H	0.11 ± 0.05	0.10 ± 0.03	n.s.

Data are expressed in μm^2 of silver deposit per μm^2 of field; specificity control values subtracted. n.s., not significant.

(means ± S.E.M. were 130.4 ± 25.6 and 95.5 ± 16.0 fmol per mg of tissue).

Without the preincubation step, the mean binding of [^{3}H]etorphine with DPDPE and U50,488H in chronic morphine-treated tissue was not significantly different from the binding after incubation with [^{3}H]etorphine and all three selective agonists. With only 1 min of preincubation, the mean binding in the presence of DPDPE and U50,488H in morphine-treated tissue had risen to 15.1% of the value in vehicle-treated tissue, and thereafter, up to the longest preincubation time tested (90 min), there was no further rise. Typical sections are illustrated in Fig. 1.

Median preoptic nucleus

Subjective assessment. Regional silver-grain distribution in sections containing the MPN, probed with [^{3}H]etorphine alone, was similar to sections containing the SON. Assessed subjectively, in control rats, the relative intensities were: white matter, 0 to +; MPN, +++; patches in the caudate–putamen, ++++; elsewhere in the caudate–putamen and in the cerebral cortex, ++. Specificity control sections, incubated with [^{3}H]etorphine and either naloxone or all three displacers, gave very weak images.

In i.c.v. morphine-treated rats the pattern of silver-grain distribution in [^{3}H]etorphine-probed sections

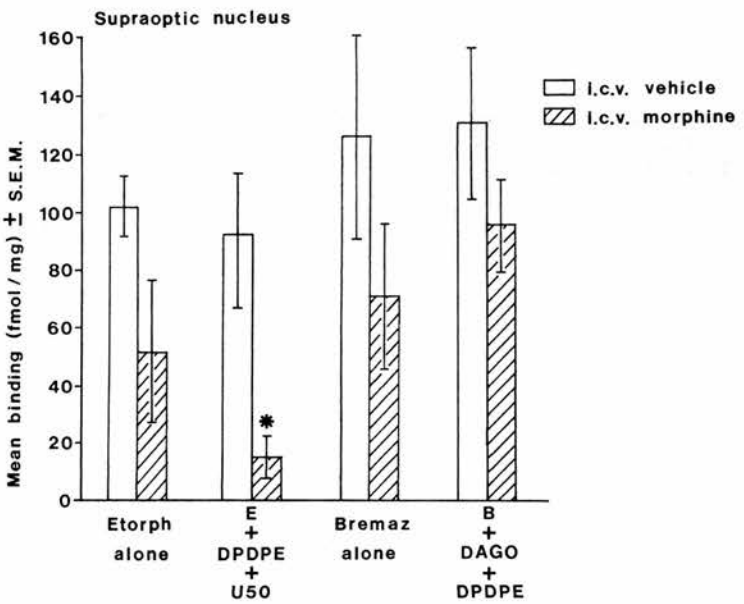

Fig. 2. Treatment group means (±S.E.M.) of ^{3}H-ligand binding to the SON of i.c.v. vehicle-infused rats (□) and i.c.v. morphine-infused rats (▨), in each incubation category. The values shown represent specific binding to opioid receptors since specificity control values have been subtracted. Values are expressed in fmol of ^{3}H-ligand per mg of tissue. Etorph alone, binding after incubation with [^{3}H]etorphine alone (without displacers). E + DPDPE + U50, binding after incubation with [^{3}H]etorphine and DPDPE and U50,488H. Bremaz alone, binding after incubation with [^{3}H](−)-bremazocine alone (without displacers). B + DAGO + DPDPE, binding after incubation with [^{3}H](−)-bremazocine and DAGO and DPDPE. *$P < 0.05$ (Student's t-test).

was similar to the control group, but the silver-grain density appeared lower in the MPN and elsewhere.

Quantitative assessment. Chronic morphine treatment significantly decreased grain density over MPN after incubation in [^{3}H]etorphine (by 29.7%, $P < 0.05$; Student's paired t-test, Table 3). Mean specific [^{3}H]etorphine-binding in the MPN was reduced by 77.0% in morphine-treated rats. In the control and morphine-treated groups the means ($\pm$S.E.M.) were 76.2 ± 9.8 and 17.5 ± 5.2 fmol per mg of tissue, respectively ($P < 0.01$, Student's t-test). Prolonged preincubation, for 90 min, before incubation in [^{3}H]etorphine did not affect the difference between control and morphine-treated rats.

Posterior pituitary

Subjective assessment. Whole pituitary sections were apposed to film, but only the PP gave an autoradiographic image. Typical autoradiographs are shown in Fig. 3. No consistent differences between central and peripheral parts of the posterior pituitary were found in any incubation category. Using [^{3}H](−)-bremazocine alone or with DAGO and DPDPE an intense image was obtained (+ + +). Inclusion of DPDPE and U50,488H in the incubating medium gave a less intense image (+ +). Incubation in the presence of DAGO and U50,488H resulted in a very weak image (0 to +). Specificity control sections, incubated with [^{3}H](−)-bremazocine and naloxone, also gave very weak images (0 to +), and chemography control sections, incubated without ^{3}H-ligand, gave no images at all.

Images from the posterior pituitaries of morphine-treated rats were similar to those from vehicle-treated controls.

Quantitative assessment. Whether the quantitative results from the posterior pituitary were expressed as mean grain densities, or as mean binding, no statistically significant differences were found between the centre, and the periphery, of the PP for any incubation category so central and peripheral data have been combined.

In control rats, the mean grain densities were highest after incubation with [^{3}H](−)-bremazocine alone (Table 3). Chronic morphine treatment had no significant effects (Table 3). Results from PP sections of control rats probed with [^{3}H](−)-bremazocine alone or with naloxone, showed that specific binding averaged 79% of the total. Mean ($\pm$S.E.M.) specific binding after incubation with [^{3}H](−)-bremazocine without displacers, was 11.6 ± 6.30 and 18.0 ± 8.46 fmol per mg of tissue in control and i.c.v.

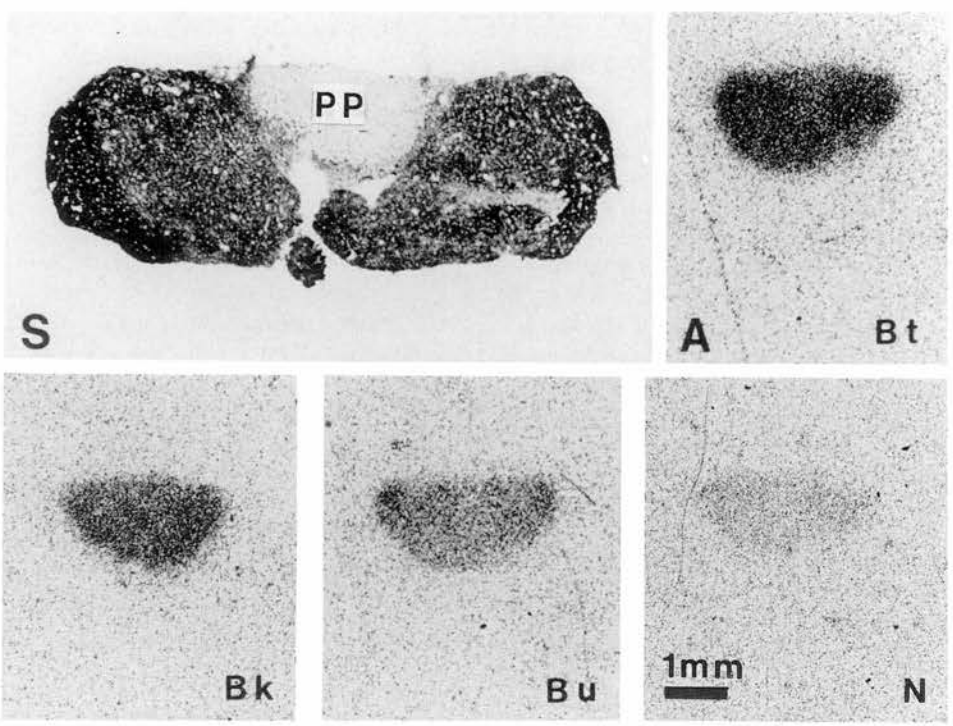

Fig. 3. (Top row) S is a 10-μm section of rat pituitary, incubated with [^{3}H](−)-bremazocine, apposed to Hyperfilm-^{3}H for 16 weeks, then fixed in acetic acid–ethanol–formalin, and stained with Cresyl Fast Violet. The PP is the pale-staining area. A is the autoradiographic image of S. Note that only the PP has produced an image. This image shows binding to opioid receptors after incubation in [^{3}H](−)-bremazocine alone (without displacers) (Bt). Magnification × 8. (Second row) Autoradiographic images of sections incubated with [^{3}H](−)-bremazocine and DAGO and DPDPE (Bk), or with [^{3}H](−)-bremazocine and DPDPE and U50,488H (Bu), or with [^{3}H](−)-bremazocine and naloxone to antagonize all binding to opioid receptors (N). Magnification × 8.

morphine-infused rats, respectively. In control rats binding was only 9.2% of that in the SON in the same incubation category. In the presence of DAGO and DPDPE the mean (±S.E.M.) values were 3.25 ± 2.09 and 2.72 ± 1.42 fmol of ^{3}H-ligand bound per mg of tissue in control and morphine groups, respectively. For control rats, this binding was only 2.5% of the level in the SON in similar conditions. Mean (±S.E.M.) specific binding after incubation with [^{3}H](−)-bremazocine and DPDPE and U50,488H was 2.00 ± 1.21 fmol (control group), and 1.07 ± 0.73 fmol (morphine group) per mg of tissue. There were no significant differences between control and morphine-treated rats for any incubation category.

Blood plasma analysis

There were no significant differences between the control and i.c.v. morphine groups for any of the parameters measured, i.e. trunk blood plasma oxytocin, glucose, or sodium concentration, or osmolality (Table 4).

DISCUSSION

Supraoptic nucleus

Control rats. Unlike some previous studies on male rats[35,36] we found ^{3}H-ligand-binding in the SON of female rats in conditions designed to be selective for μ-type opioid receptors. We suggest that this represents binding to μ-type opioid receptors, for the following reasons. Firstly, [^{3}H]etorphine is a broad spectrum ^{3}H-ligand for opioid receptors, but with a 1000-fold molar excess of the unlabelled agonists, DPDPE and U50,488H, known to have high relative affinity for δ- and κ-type receptors, respectively,[16,40] competition for binding sites would be expected to leave predominantly μ-receptors available for [^{3}H]-etorphine. Although U50,488H is a stronger displacer at κ-type opioid receptors of the guinea-pig (postulated type 1 κ-receptors) than at κ-receptors of the rat (postulated type 2 receptors),[62] it nonetheless blocks *in situ* [^{3}H](−)-bremazocine-binding to rat κ-receptors.[38,39] Secondly, incubation with DAGO (which has a high relative affinity for μ-receptors), in addition to DPDPE and U50,488H, reduced [^{3}H]etorphine-binding in the SON to a very low level. Thirdly, elsewhere in the sections containing SON profiles were intense patches of labelling, present after incubation in media designed to demonstrate μ-receptors, but absent after incubation in media designed to show δ- or κ-receptors. Other authors have claimed these intense clusters in the caudate–putamen to be μ-receptors.[57] Fourthly, the change in the SON during chronic morphine treatment was confined to μ-selective binding. This suggests that the subtype demonstrated by this selective incubation differs from the others, and that it is uniquely responsive to a known μ-selective agonist (morphine). Lastly, the demonstration of μ-type opioid receptors in the SON is consistent with the inhibitory action of morphine on the electrical activity of oxytocin neurons when applied close to their cell bodies.[49,58]

Specific binding to opioid receptors was also demonstrated in the SON after incubation with [^{3}H](−)-bremazocine in the presence of DAGO and DPDPE, a procedure designed to be selective for κ-receptors. In the caudate–putamen, this type of incubation did not show the intense patches of binding shown by ^{3}H-ligand alone, or by incubation in conditions intended to be μ-selective; it seems likely that DAGO and DPDPE prevented binding to the receptor subtype(s) at these clustered sites. Addition of U50,488H, a known κ-receptor-selective agonist,[16] to the other two unlabelled selective agonists reduced [^{3}H](−)-bremazocine-binding to a low level both in the SON and caudate–putamen, supporting the assumption that κ-receptors were predominantly involved. Demonstration of κ-receptor in the SON is in agreement with previous reports,[35,36] and with electrophysiological studies showing inhibitory actions of U50,488H on oxytocin (and vasopressin) neurons in the SON.[49] Indeed, suppression of electrical activity by U50,488H, and by morphine, has even been achieved on the same supraoptic neuron (Russell *et al.*, unpublished observations).

With the low resolution of the techniques used, we were unable to determine the distribution of μ- and κ-receptors on or between neurons,[24] or indeed whether they had been internalized.[29] It is possible, by analogy with the PP gland,[12,33] that some of the receptors were on glial cells, especially astrocytes.

Effects of intracerebroventricular morphine infusion. In the SON, binding of [^{3}H]etorphine in conditions designed to be μ-selective was reduced by over 80% by chronic morphine treatment compared with control rats. Morphine did not affect κ-selective [^{3}H](−)-bremazocine-binding.

One possible explanation for decreased binding of [^{3}H]etorphine to the SON after chronic morphine treatment could be that morphine still in the tissue

Table 4. Means (±S.E.M.) of data from blood plasma samples

Treatment group	Oxytocin concentration (pg/ml)	Glucose concentration (mM/l)	Sodium concentration (mM/l)	Osmolality (mOsmol/kg)
i.c.v. vehicle-infused ($n = 6$)	13.23 ± 5.06	7.82 ± 0.43	138.29 ± 2.23	270.00 ± 5.63
i.c.v. morphine-infused ($n = 6$)	8.67 ± 1.77	7.31 ± 0.24	142.71 ± 1.41	268.29 ± 5.99

competes with [^{3}H]etorphine. Morphine is remarkably persistent in brain after peripheral administration, [^{14}C]morphine still being detectable three weeks after a single injection,[37] however, morphine can be washed out *in vitro*.[59] Similarly, altered release of endogenous opioid in i.c.v. morphine-infused rats might compete with [^{3}H]etorphine. However, etorphine has a high affinity for opioid receptors,[22] and 5′-guanylylimidodiphosphate was included in the preincubating medium to encourage dissociation of receptor-bound opioids,[12] and we showed that preincubation for much longer than the 15 min used did not result in any increase in ^{3}H-ligand binding. It seems reasonable to conclude that *in vivo*, the number of μ-receptors in the SON is reduced by chronic i.c.v. infusion of morphine, and that this reduction explains the decreased binding of ^{3}H-ligand. However, a possible wash-resistant blockade by some of the i.c.v. infused morphine, cannot be ruled out. A significant reduction in [^{3}H]etorphine-binding was found also in the MPN, though not resolved to a particular opioid receptor subtype.

A reduction in number of available μ-receptors might allow SON oxytocin neurons, and their inputs, to function normally in morphine-treated rats, as indicated by similar concentrations of oxytocin in trunk blood plasma from the i.c.v. morphine- and vehicle-treated rats in the present study. Normal functioning has also been inferred in previous studies.[6,43] The consequence of a partial loss or blockade of μ-type receptors would be to decrease the pool of spare receptors.[14,32,53] Thus, even if the affinity of the receptors for morphine were unaffected, this would decrease the number of receptors which could be activated at a given concentration of morphine. This effect would contribute to tolerance, and the extent of the loss of binding we have measured might almost be great enough to explain morphine-tolerance[15] in the female rat oxytocin system. We found no evidence after i.c.v. morphine infusion for changes in trunk blood plasma glucose or sodium concentrations, or osmolality, that might stimulate the oxytocin system through osmoreceptor mechanisms,[47] and perhaps alter opioid receptor density in the SON.

Posterior pituitary

Control rats. In common with earlier work,[51] we found a much lower density of opioid receptors in the PP than in the brain. Longer exposures were required to produce autoradiographic images. Most binding was obtained after incubation with [^{3}H](−)-bremazocine alone, or in the presence of DAGO and DPDPE, which was intended to be selective for κ-receptors. Since addition of U50,488H to DAGO and DPDPE reduced [^{3}H](−)-bremazocine-binding to a low level, it is probable that κ-receptors were indeed responsible for most of this binding. Other authors have also suggested that opioid receptors in the PP are predominantly of the κ-receptor subtype.[9,12,25] In the presence of DPDPE and U50,488H, which was intended to be μ-selective, there was still some binding by [^{3}H](−)-bremazocine, but there was less binding in pilot studies with [^{3}H]etorphine so the presence of the μ-receptor subtype in the PP remains controversial.

We did not find any differences in opioid receptor density between the central and peripheral zones of the PP of female rats, unlike the studies of Herkenham *et al.*[25] and Brady and Herkenham[9] on male rats.

Effects of intracerebroventricular morphine infusion. Although i.c.v.-infused morphine reaches the PP,[43] we detected no change in [^{3}H](−)-bremazocine binding in any incubating conditions. The lack of effect of morphine on κ-receptors in the PP is consistent with the persistence of endogenous opioid- (probably dynorphin)-inhibition of oxytocin secretion in isolated PPs from i.c.v. morphine-infused rats *in vitro*.[3] Thus, chronic morphine treatment neither affects the number of κ-receptors, nor induces cross-tolerance to the endogenous ligand in the PP. However, the density of κ-receptors in the PP can show plasticity: during dehydration for five days the number of κ-receptors decreases by about 40% without a change in affinity.[11]

CONCLUSION

The data we have on the oxytocin and oxytocin mRNA content of SON neurons, their firing rate, and the responsiveness of their terminals in the PP to electrical stimulation, all indicate that the synthesis of oxytocin and the coupling of electrical activity to secretion are not altered in morphine-tolerance.[3,6,43,54] This leaves opioid–receptor interaction, and early post-receptor events regulating electrical excitability, as likely sites for tolerance. We have provided evidence in this study for a decrease in number of available μ-receptors in the SON, and of available opioid receptors in an input pathway, the MPN, which regulates oxytocin neurons, but we have not distinguished intracellular forms from surface forms of the receptor. There may also be reduced effectiveness of remaining receptors available for binding to ligand because of attenuated coupling of receptor to a $G_{i/o}$-like protein, as has been described for morphine-tolerant locus coeruleus neurons,[15] but this has not been investigated in SON neurons.

Morphine-dependence in the oxytocin system, revealed by injection of naloxone, is expressed as withdrawal excitation of oxytocin hypersecretion resulting from a large increase in the firing rate of oxytocin neurons.[6,30] This phenomenon is mediated through μ-receptors,[31] which the present study has shown to remain available in the SON at about 20% of the number in morphine-naive rats. It is difficult to explain withdrawal excitation of oxytocin neurons in terms of a reduced number of available μ-receptors. Adaptive changes in post-receptor mechanisms regu-

lating ionic fluxes seem more likely. There may also be compensatory changes in non-opioid pathways.

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